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TITLE: ATOMIC FORCE MICROSCOPY STUDY OF UROLITHIASIS EVOLUTION IN SPACE CREWS DURING LONG-TERM MISSIONS

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Overview

Urolithiasis is a common disease on Earth. In the last decades, it has been observed that this problem also appears during and after space flights. One of the most remarkable changes of this phenomenon is the frequency of cases onboard and on Earth. While in our society it affects approximately the 7% of the people, it has been discovered that onboard it affects the 18% of the astronauts. Analyzing this variation, it is possible to confirm that this problematic is really relevant.

This project analyzes the different aspects related to urolithiasis such as the epidemiology, the pathogenesis or the composition of renal calculi. Moreover, it collects and studies the existing information about this problem during space missions. It puts an special emphasis in the controversy that exists about CNP-like particles. Then, the experimental part of the project begins. Some real samples of calculi are analyzed with the interferometer, the atomic force microscope and the field emission microscope. The images obtained from these techniques are analyzed with an image processing software.

The results obtained from these studies define that all the analyzed samples have the presence of CNP-like particles. Their dimension is between 30 and 200nm. They are located as independent particles and also as aggregations. Moreover, they have similar characteristics and an aspect similar to the ones defined from different scientists. To verify the results, the samples are analyzed with the field emission microscope which shows the same kind of particles in different crystals from all samples.

Finally, the information recompiled was also exposed as a part of a possible Mars reference mission. Currently available proposals do not take into account urolithiasis and through this project is possible to see its importance for a long space mission.

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Introduction

With the potential for long-term missions around the Earth's orbit, the Moon and Mars, some health issues such as the physiological effects of microgravity and radiation become a major concern for space crews. Long periods of microgravity are known to reduce bone density, muscle strength and cardiovascular fitness. The calcium which is lost from bones can end up in kidneys, form kidney stones (KS) and eventually put astronauts at risk. For instance, 14 American astronauts suffered from urolithiasis in the period between 2001- 2006[1]. These mineral deposits can travel through the urinary tract, causing intense pain. One of the most common types of KS is caused by the buildup of calcium oxalate, although calcium phosphate is also common [2].

When a renal stone appears during a space mission apart from provoking hard pain to the crewmember who suffers from it, it can also create huge problems to the correct development of the whole mission. This statement is based in the fact that the pain of nephrolithiasis can be so severe and it can disable the astronaut to do some tasks of the mission such as piloting the spacecraft or performing extravehicular maintenance. Moreover, a current nephrolithiasis can produce a lot of different complications which are easily solved on Earth but not onboard for several reasons. For example, a surgical treatment cannot be done. A good example is a forniceal rupture, which can cause an urosepsis if it is not solved in a short period of time. For this reason, a renal stone can advance the return of the space mission without finishing its principal objective.

During this project urolithiasis is analyzed from different points of view, the obtaining of its basis, the understanding of the problem on Earth and the comparison of this information with the data obtained onboard from a space mission. The main problem is that the information related to urolithiasis on board is very poor. However, this information is useful to define a such relevant concept as it is the relation between microgravity and calculus. Nowadays, there is huge debate about the nanobacteria theory, which is an important issue for this project. Once the relevant points of urolithiasis on board are defined, they are focused on the experimental part of the project. It consists on an analysis of the calculus using three different techniques: interferometry, atomic force microscopy and field emission scanning electron microscopy.

A complex experimental analysis process is performed in order to achieve the significant results which are discussed and contrasted on this thesis. One of the main conclusions obtained from this project is that there is presence of CNP-like particles on all the samples. Moreover, their dimension is between 30 and 200nm which is similar to the ones obtained from other scientist specialist on this topic. The location of these particles is also characteristic because they are found as independent particles and as aggregations. Furthermore, the results obtained with the CNP-like particles are related with the once that are creating big controversy in the scientific community.

Finally, it can be concluded that all this information would be relevant if a long space mission was done. Therefore, it is proposed as a part of a Mars reference mission to help to avoid this problem on board.

1. Objectives

Astronaut's health is one of the main preoccupations of space agencies when they consider the possibility of a long space mission. There are several topics involved with this problematic. Apart from radiation, one of the most important and relevant is the effect of microgravity on the human body. It is known that this condition affects different parts of the body such as the skeleton and heart. However, another part which is also affected, it is the genitourinary system. This problem is not directly provoked by these conditions; it is a secondary effect of the bone degradation.

This project studies of a concrete genitourinary problem, which is the urolithiasis. This disease is more common onboard than on Earth. Moreover, it is most difficult to be treated as the chirurgical options have to be discharged. Therefore, it is really important to understand this phenomenon in detail and also to define different ways to avoid it and how to solve this situation.

There are different objectives in this project. The first one is to define the concept urolithiasis. Also, it is important to know the characteristics of the calculi and also the reasons why they appear.

The next objective is moving this problematic onboard of a spacecraft. This means that all the data in relation with urolithiasis during space mission have to be collected. Moreover, it is important to analysis the experiments that the different space agencies performed in the last years to understand this disease.

Once the problem has been clearly defined, the next objective is to analyze in detail samples from real patients from Earth. Different techniques will be used to achieve this objective such as interferometry, atomic force microscopy and field emission microscopy. Using these techniques, it will be possible to analyze in detail the topography of the stones.

Finally, the last objective is to obtain conclusions of the results by comparing the information obtained from the experimental analysis of the stones and the information previously collected from this problematic.

In summary, the main objective of this project is to analyze experimentally samples from real patient and define the results in relation with the problems that the astronauts will have during a long mission, for example, to Mars.

2. Urolithiasis

Urolithiasis can be defined as the clinical condition where the formation of crystal aggregates in the urinary tract results in kidney stones. The presence of a kidney stone in the human body can be externalized in very different ways, it can produce no symptoms but it can be associated with several pain caused for one or some of the following symptoms: gross or microscopic hematuria, obstruction of one or both kidneys, and urinary infections.

Urolithiasis is considered one of the most common medical problems in the present society as it affects a high percentage of people but this illness exists since antique societies. However, its characteristics have changed during the years. For instance, the site of stone formation has moved from the lower to upper urinary tract. Another visible change was the variation in the gender of the affected people. Although in the beginnings the disease was limited to men, nowadays it also affects to women. Furthermore, the treatments also evolved and will be improved in future times. The treatments are minimally invasive and even there are ones which are non-invasive. This advanced techniques facilitated the way how stones are removed. Another clear data which shows the importance of this disease is the increase of the treatments and the increase of money involved with them. In the last 10 years, the diagnosis of urolithiasis was increased approximately by a 50%. [3]

2.1. Epidemiology

Kidney stones are a common illness in the westernized countries. The annual incidence can vary from 0.5% until 1.9% and the lifetime incident rate is approximately 13% in men and 7% in women. Moreover, this problem is most common in middle-age people as the most common age when it is possible to be affected is around the 45 year old in men and around the 41 in women. The values expressed before are during the period where it is more present, if the age group considered is people older than 70 years or younger than 30, the values of lifetime incident rate change as they are not the groups more affected. Besides, if some non-westernized countries are taken in account, such as a Middle East country, these parameters also change. The variation can be given to the greater sunlight which increases losses of body water and urine concentration. This age group and countries were chosen because they are coincident with the astronauts who are the base of this study case.

Apart from the age, gender and geography, there are some other aspects which also affect the presence of kidney stones, for instance race, weight and body mass index. Studying the race parameters, the most affected are the non-Hispanic whites, they are over racial groups such as non-Hispanics blacks or the Mexican Americans. The previous ones are the extremes as in the middle it is possible to find the Hispanics and Asian.

Men and women who are obese have a greater risk to suffer urolithiasis. People who have a weight higher than 100 kg have more possibilities to create a kidney stone than those who are slimmer. It is also possible to do this comparison with the mass body index. If this parameter is greater than 30 it is easier to create a kidney stone than if it is close to the 22, which is the appropriate value.

In summary, kidney stones occur more commonly in men than women by a ratio of 1.73. Moreover, whites have highest incidence of stones compared with Asians, Hispanics and African Americans. Prevalence of stone disease shows geographic variability, with the highest prevalence of stone disease in the Southeast. Finally, the risk of stone is directly related with the weight and the body mass index of the patients. [3,4]

2.2. Pathogenesis

The physical process of stone formation is a complex cascade of events that occurs as the glomerular filtrate traverses the nephron. To understand this process, it is important to imagine the solution as water which contains calcium oxalate crystals.

When these crystals do not grow and the concentration does not change, the solution is considered stable and all the system is in equilibrium. The variation of these crystals depends on the free ion activity as the crystal will dissolve or they will grow in relation with it. If the system is moved from the equilibrium and the ion activity is raised the crystals will grow. In the moment when this process begins, the appearance of the urine is the same. It is possible to consider this moment as a phase, called metastable. If the ion activity continues increasing, new crystals will appear. At that point, the formation of new products begins. Until this point, urine becomes unstable with respect to stone-forming salts and it will begin to form crystals or nuclei.

Unstable urine does not always create nuclei or new crystals as the presence of urinary inhibitors is also so important. Nephrocalcin, uropontin, and Tamm-Horsfall protein are important inhibitors of crystals nucleation, growth or aggregation.

On the other hand, if the crystals grow, they can flow out with the urine or become retained in the kidney. The stones need anchoring sites that help growth and aggregation to create the stone. A good example is the calcium stones which can originate from subepithelial plaques composed of calcium apatite. They act as an anchor on which calcium oxalate stones can grow. [3,4]

2.3. Composition of renal calculi

There are different components from which the renal stones can be made and they can also be made from a mix of them. However, the most common one is calcium which is principal element in nearly 75% of stones. On the other hand, calcium oxalate makes up about 60% of all stones and mixes of calcium oxalate and hydroxyapatite are the 20% of the total. Brushite stones are less common as they are only the 2%. Both uric acid and struvite (magnesium ammonium phospahate) stones occur approximately 10% of the time. The rarest are the cystine stones which are 1% of the samples. Stones associated with medications and their products such as triamterene, a denosines, silica, indinavir, and ephedrine are very uncommon and usually preventable. In the following picture (Fig.2.1) it is possible to see the different aspects of this stones. [3,4,5]

Fig. 2.1 Images from different renal calculi (http://www.herringlab.com)

2.3.1. Calcium based kidney stones

Calcium stones are the most common ones, therefore, the samples used for this study are of this kind. Calcium based stones can be evaluated based on complete 24-hour urine collections and the results can be divided into four different groups. However, a patient may have one or multiple of these abnormalities. They can be defined as [6]:

- Hypercalciuria: Excessive urinary calcium.
- Hyperuricosuria: Abnormally high concentration of uric acid in the urine.
- Hyperoxaluria: Abnormally high concentration of oxalate in the urine.
- Hypocitraturia: Abnormally low concentration of citrate in the urine.

The abnormality which will be studied in depth is the hypercalciuria as is the one related with the problem of astronauts as there are microgravity conditions and it provokes bone mineral losses. [7]

From a medical point of view; it is possible to detect a hypercalciuria if during a 24-hour urine collection there is more than 4mg/kg of calcium in urine. The most common reason where hypercalciuria appears is associated hyperparathyroidism or sarcoidosis, with or without hypercalcemia.

However, sometimes hypercalciuria occurs with normal serum calcium and in the absence of any systemic diseases, and it is called idiopathic hypercalciuria. This kind of hypercalciuria is related with this study case.[8]

Hypercalciuria also can be subdivided in three other groups depending of the origin of the excess of calcium in the urine[3,4]:

- Absorptive hypercalciuria: it is caused by one of several mechanisms that increase calcium absorption from the digestive tract. There are three types of this hypercalciuria and once can be modified with some diet and others are more complex.
- Resorptive hypercalciuria: This high urinary calcium concentration is caused by the reabsorption of existing bodily stores such as bone.
- Renal hypercalciuria: it is caused due to a defect in the kidney that causes excessive calcium loss. This problem requires medical therapy.

2.4. General therapy for urolithiasis of Earth

As it was explained before, the urolithiasis is a recurrent problem. To avoid this situation, it is important to measure urinary supersaturarion and analyse the samples to determine which kind of stone the patient has. The patients who already have urolithiasis or who have a strong familiar history related with this

problem follow some recommendations to change the fluid and dietary habits to reduce urinary supersaturation to prevent future stone formations. Due to these variations without pharmacologic therapy, stone recurrence rates have been 60% over 5 years. Therefore, as the crew of space flight has more possibility to have a kidney stone, thought, they do not have previous experience; the same countermeasures have to be taken into account when they are onboard [3,4].
They can be summarized in the following points:

- Increase in fluid intake: it will increase urine volume.
- Restrict the dietary sodium: it will reduce urinary calcium excretion.
- Restrict the animal protein: it will reduce urinary calcium excretion and will increase the excretion of the calcification inhibitor citrate.
- Ingest an age and gender appropriate amount of dietary calcium.
- Restrict the oxalate consumption.

Two of these points are more difficult to realize onboard than on Earth. The first one is the increase of fluid volume intake because the astronauts suffer space motion sickness. The other one is the restrictions in dietary sodium. The reason why it can not be done is because the food-prevention techniques use this element.

On the other hand, the use of some pharmacological elements can be really useful in this case because they can raise the urinary volume and also can prevent the reduction of the pH of the urine and the citrates[9-13].

3. Aeromedical significance of kidney stones

It is known that a prolonged exposure to microgravity causes a number of physiologic changes. The most frequent ones are musculoskeletal degeneration with the potential loss of muscle and bone mass, regional bone mineral losses showed by hypercalcemia and hypercalciuria during and after spaceflight, and cellular/molecular alterations in immune system function[14,15].

Apart from the changes in the musculoskeletal and the immune system, the genitourinary (GU) system is also commonly affected during spaceflight. To reinforce this information, it is possible to say that 10% of astronauts participating in Shuttle flights between 1981 and 1998 reported GU symptoms during flight. The information related with renal calculus, concretely, in the number of astronauts affected during/after a space-flight varies considerably depending of the information source. The number of American astronauts affected during the period of 2000-2006 oscillates from 11 to 14. Although, the variation is quite large, the importance is that there is a presence of this problem. Doing a little statistic, it is possible to observe that during this period there were a maximum of 87 American astronauts, taking into account that some of them flight in more than one occasion. That means that around 18% of the total crew members were affected during or after the flight for urolithiasis. For this reason, this is a significant problem which has to be studied and it is important to find a solution. [14-16]

4. Influence of urolithiasis on space mission

There are several factors which can affect the human body during a space mission for instance microgravity or radiation. It provokes several changes in the organism such as fluid balance or musculoskeletal degradation. However, there are other aspects which affect the formation of calculi which are more related to the station and the schedule than to the microgravity effects; these are the different problems related to voiding. In the next section the previous reason for the increment of kidney stones will be explained in depth. [14]

4.1. Body fluid balance

The distribution of the different fluids inside the human body is clearly related with the force of gravity, for this reason, when the astronauts are in microgravity conditions body fluids are redistributed. This redistribution also disports the body's volume sensors. They perceive the resulting shift of fluids as an overload. An easy way to seen this fluid shift is observing the facial puffiness and nasal stuffiness.

Another clear experience related with body fluid redistribution produced by the microgravity is the space motion sickness. This problem causes vomits and a diminution on the thirst feeling. Therefore, the final volume intake is clearly reduced. The combination of fluid redistribution and decreased fluid intake reduce urine output 72 hours after arriving in microgravity. However, this reduction can be observed during the all mission, facilitating the appearance of urinary calculus due to increased urinary solute concentrations and osmolality. [14]

4.2. Bone Mineral Loss

Long-duration mission provokes bone mineral loss as their gravity-resistance is reduced close to zero. Space flight has a reasonable analogy with bed rest as their bone physiology and calcium kinetics are similar. This semblance helps to develop countermeasures for musculoskeletal degradation during long-duration space flight in relation with physical exercise and the utilization of different pharmacologic agents. [18-20]

On the other hand, there are some difference between the astronauts and the patient which rest on bed such as rate and magnitude of the demineralization of the key regions. Some information obtained from the joint Shuttle-Mir flights showed that this regions are normally trochanter, femoral neck, lumbar spine, and calcaneus, thought, there are significant individual variability. Even though, they show clearly that the bone mineral density which is lost monthly is 1.3-1.5 %. [14]

Obtaining of this kind of information is extremely valuable as the studies done in Skylab and Mir were really helpful to create some countermeasures to reduce bone mineral loss in the crewmembers of the ISS. [7,8]

4.3. Voiding Issues

A mission schedule has sometimes an intense operational timeline provoked that some necessary actions such as eating, drinking, and voiding are delayed or skipped. These stressful periods reduce the intake of fluids and sometimes may cause a clear dehydration to the astronauts. This reduction of fluids intake and voiding increases the urinary solute concentration, which also increase the possibility to create a renal stone. Apart from this problem, there are other sicknesses related with the genitourinary (GU) system which can also appear such as infections, bladder calculi or urinary retention. [14]

4.4. First studies of neptholithiasis during space flights

Firstly, the cosmonauts' history will be analysed. During the history of the Russian space program, three cosmonauts had post flight urinary calculi. None of them had any evidence of urolithiasis during the periods of preflight or inflight. Obviously, the stones were not detected before space flight. Moreover, the cosmonauts were evaluated after the critical period and none of them had any anatomic or metabolic abnormalities justifying the formation of the calculi.

In one occasion, one cosmonaut developed a supposed (not indentified on board) renal stone during a long duration mission. The crewmember suffered an intense pain. Therefore, this unexpected situation created important problems in the development of the mission's tasks and in the in-flight timeline. The urolithiasis was solved spontaneously in a few days and finally the mission was completed.

The next step will be the analysis of the American space mission. The first studies for the metabolic changes, which produce renal stones, where during the three Skylab missions in the 1970s. They performed a set of studies during pre-flight, in-flight and also in the post-flight period.

During the in-flight studies, they observed an important decrease in fluid intake which directly affected the urine volume during the first 6 days of the mission. Apart from the variation in the volume of the intake/outtake fluids, there were changes in the biochemistry of the urine. There was an increase in the extraction of calcium, sodium, potassium, chloride, phosphorus, and magnesium and a decrease of uric acid. These changes provoked a situation optimum for the creation of renal stone as there were less urinary volume and a high concentration of the salts which normally crystallize.

During the first few hours of landing, they appreciated some changes in the urine chemistry which favour the risk of calcium oxalate and uric acid stone formation. Comparing this information with the pre-flight information, it was possible to observe an increase in the concentration of salts in urine and a decrease in urinary inhibitors.

Due to the probability of calcium stone formation, a concrete group of calcium balance experiments were realized. The results of these studies showed a rate of daily calcium loss of 200-300mg/day in the 28-day (Skylab2), 59-day (Skylab 3) and 84-day (Skylab 4). As it was explained before, this loss of calcium can be easily related with hypeparatiroidism. For this reason, they did an analysis of this hormone. The results showed no variation in comparison with Earth values. Therefore, the conclusion which can be extracted of these studies is that the increase of calcium in urine is directly provoked by the leach of bone calcium from the skeletal system due to diminished bone loading in microgravity more than any other disease.

Moreover, they did a daily phosphorous balance study. The surprising results of these experiments were obtained during Skylab 2 and Skylab 4. The daily phosphor loss rate varied from 222 to 400 mg/day and, due to an unknown reason, during Skylab 3 the loss rate was much lower.

Finally, they continued the investigation during post-flight. It was observed that during the 6 days after the landing the levels of sodium, potassium chloride, phosphorous, magnesium, and uric acid were reduced. However, the values of calcium continued as high as in pre-flight values. However, in 14-18 days the urinary components returned to the pre-flight values in all the cases. [7,14]

4.4.1. Astronaut urinary analysis

In the following table, it is possible to see a clear example of a real urine analysis of an astronaut during a NASA mission. NASA has compiled a large database with space research and Earth-based clinical research which helped them to create some theories to avoid and understand the creation of kidney stones during and after the space flight. However, there are some weak points to create a complete theory.

Table 4.1 information from principals of medicine for space flight[14]

Parameter		Preflight	Flight day <20	Flight day <30	Flight day <100	Flight day >100	Postflight +0	Postfligt +7	Postflight +14
Total Volume	(L/day)	1.79	1.043	0.873	1.109	1.038	0.54	0.73	1.85
Calcium	(mg/day)	211	255	245	436	338	224	113	161
Oxalate	(mg/day)	35.77	13.1	17.7	38.6	39.9	20.5	27.2	35.5
Uric acid	(mg/day)	676	376	632	659	571	355	523	518
Citrate	(mg/day)	691	610	962	1119	765	618	895	1043
pH		5.93	6.14	5.97	5.49	5.34	5.22	6.18	6.28
Sodium	(mEq/day)	221	141	183	269	158	136	211	119
Sulfate	(mmol/day)	25.0	25.6	22.4	34.3	25.4	23.5	14.6	10.3
Phosphorus	(mg/day)	802	881	1110	1409	1163	544	797	694
Magnesium	(mg/day)	139	175	132	199	169	109	87	142
Potassium	(mEq/day)	79	80	73	96	80	64	57	101
Creatinine	(mg/day)	1701	1841	1910	2160	1946	1408	1507	1717

Some of these values and variations are clearly understood. For instance, the decrease in daily urine output is mainly produced by the reduction fluid intake. That is also related with space motion sickness which provoked vomits and a diminution on the thirst feeling. Besides, it was explained in section 2.3.1., microgravity exposure causes losses of calcium which produce a resorptive hypercalciuria. Apart from the saturation of calcium, it is possible to see that there is a saturation of all the other different salts. Furthermore, citrate and other inhibitors of renal stone formation also decreased during space flight, especially in the first days and also immediately after the landing. All these changes increase the risk of renal stone formation. [14]

For these reasons, NASA says that the risk could be decreased with the increase of fluid intake until the daily urine output arrives to 2 l/day. [9,14]

4.5. New studies of neptholithiasis during space flights

A lot of experiments are being done in relation with the body changes during space missions. Moreover, several of them are related with the formation of kidney stones due to microgravity effects. This topic is in the agenda of all space agencies but they raise the problem from different points of view. The range of experiments goes from the analysis of different ways to avoid the creation of renal calculi until studies which simply try to clarify the reason why they appear.

4.5.1. Bed-rest experiments

M. Monga from University of Minessotta did a group of experiments related with the body changes in simulated microgravity. Those were based in a group of 11 sets of identical twins remained on simulated microgravity. To create this false microgravity the twins were put on 6-degree head down in tilt beds and they rested for 30 days in this position.

The experiment consist that one twin per pair was randomly selected to do exercise in supine position. They did these exercises in a special chamber where the pressure was in a lower body negative. They did this process 6 days every week during 40 minutes. Moreover, they also rested 5 minutes more inside the chamber in a negative pressure of 50 mm Hg. On the other hand, the other twin served as a non-exercise control.

From these studies, Monga obtained some significant results in just one week. They observed that urinary concentration of calcium was clearly changed in the twin who rested on bed without realizing any exercise. Moreover, the other group did not have any significant change in their urine concentration.

The conclusion of this experiment is that during a prolonged bed rest, the urinary salt concentration varied in a way that promotes kidney stones formation. Moreover, if some exercise was realized in a lower body negative pressure this risk could be decreased as the risk of hypercalciuria is reduced. [2,21]

4.5.2. Experiment with potassium citrate

As it was explained before, potassium citrate is a common preventive treatment for renal stones on Earth. Concretely, it avoids the crystallization and the development of the stone. Citrate also makes the urine less acidic, reducing the risk of uric acid stones.

During STS–105, NASA decided to study the effects of potassium citrate during a real space flight. To perform the test, each crewmember received a packet of pills, one of them was pills of potassium citrate and the other was placebo. The crewmembers did not know which ones were taking. They take two pills at dinnertime. The experiment began three days before launch and it ended 14 days after landing.

Apart from recording the taken pills, they registered everything they ate or drank, when they did exercise and what medication they took. They provided samples of the urine using specific urine collection kits. The information complied was analysed after the flight. NASA measured how much urine each crewmember voided and examined the chemical composition of the urine of the crewmembers. Besides, they also checked the urine's pH, to see if the potassium citrate raised the pH to a less acidic value.

The STS–105 data will be compared with results obtained from other missions in the ISS. Through the final results, the problem of urolithiasis during space flight was better understood. Moreover, it proved the effectiveness of potassium citrate. [22]

4.6. Studies about calcifying nanoparticles

Neva Ciftcioglu is currently studying in NASA the weak points of the theory that explains the creation of kidney stones during a space flight. The experiments of Ciftcioglu et al. show that, apart from the hypercalciuria provoked by the degradation of the muscoskelete, there are other factors involved in this process because the pathophysiology of renal stones cannot be explained by crystallization process alone. [23]

Her previous investigations showed that there are calcifying agents called calcifying nanoparticles (CNPs) or nanobacteria which could have an important role on the formation of kidney stones. She is involved in the study of CNP since 90s and she has done several experimental campaigns. Her hypothesis is that this nanobacterium provokes calcium phosphate-carbonate deposition in the kidney. Some important data from other investigators such as F. Grases et al. shows that the 90% of calcium oxalate stones contain small amounts of phosphates at the calculi core. This agrees with the theories of Dr. Ciftcioglu. [24]

Neva Ciftcioglu did an important group of experiments in the University of Kuopio in collaboration with E.O. Kajander. They analyzed 72 kidney stones from Finnish patients. One of the most relevant results that they obtained from their experiments was that 97.2% of the kidney stones analyzed contained the supposed nanobacteria. Moreover, they analyzed kidney stones from different groups such as calcium oxalate, calcium phosphate, uric acid, calcium hydrogen phosphate-dihydrate, magnesium ammonium phosphate hexahydrate and all of them had small amounts of apatite. Their experiments continued and they decided to isolate the supposed nanobacteria from the real kidney stones and she analyzed them in vitro. The surprising result was that the nanobacteria created stones in vitro. An external contamination was rejected because they created some control cultures and they remained without any change. [25]

In 2005, NASA was involved in a group of experiment to study the characteristics of CNPs. One of the most relevant results was obtained when nanobacteria were conducted in a bioreactor chamber that simulates microgravity environment. This experiment showed that nanobacteria increased their multiplication rate under simulated microgravity conditions. They multiplied five times faster than in normal gravity conditions. [26]

At the present time, she is doing experiment with kidney stones from real astronauts to define if they have supposed CNP or not. She did a study of the kidney stone of an astronaut of 40 years old without any previous infection or urinary abnormality. On that calculi appeared CNP-like spherical apatite units. [27]

Apart from the works of Neva Ciftcioglu, there are other investigators which also work in this topic. They have some hypothesis which coincide with the ones created by her and some other ones which are completely opposed to her. One of the big controversies of these hypotheses is if the nanobacteria are living organism or not. A good example is G. Aloisi from University of Lyon. [28]

His hypothesis is based on the idea that there are nanobacteria involved in the process of nucleation of kidney stones. This CNPs measure between 50 and 200nm and they are in the core of the kidney stones because the majority of calculi have apatite in their core. However, they thought that these nanobacteria are not alive and they are a by-product of the living activities of bona-fide bacteria inhabiting the kidney stones. He said that the 16SrDNA found in the CNP analyzed by Neva Ciftcioglu could originate from a contaminant organism. Therefore, as the acid nucleic from CNP is not sequenced yet, they cannot be defined as live organisms. [28]

His hypothesis was that if a sulphate-reducing bacterium is immersed in a supersaturated culture medium, produces organic globules which calcify significantly. The end product of the bacterial mineralization process has several similarities with the CNPs defined by Neva Ciftcioglu. They have approximately the same size, an organic nucleus, a calcified mineral shell rich in phosphates and the ability to form colonies. [23]

Another hypothesis about the origin of CNP was developed by J. Martel et al. who based their theory in the idea that certain chemical substrates may adapt the appearance of simple microorganisms. They did a series of experiment with CaCO3 precipitates and they found that these precipitates have a similar size to CNP. Moreover, there are other similarities such as the membrane-delineated vesicular shapes, the cellular division-like formation or the aggregations in the form of colonies. [29]

As it is possible to see, there is a big controversy in this topic and if CNP-like organisms are the base of the nucleation of kidney stones and other human diseases it is really important to study this kind of particles.

5. Experimental methodology

5.1. Sample preparation

There were different processes of handling during this project as the samples have to be prepared in different ways depending of which kind of microscopy will be used to do the analysis. Firstly, the samples were analyzed without any process in the optical microscope, to use this instrument samples were put over a glass holder which was located inside the microscope. At that moment, it was seen that the samples were too big to be analyzed with the other techniques. Therefore, they were broken in new small samples while trying to maintain the flat surfaces intact as they were the parts which will be studied. The flat surfaces were considerably small, therefore, it was really important to avoid reducing more their size.

Then, the new samples had to be glued to metallic holders which are specific for the atomic force microscopy, though, they also can be used in the interferometer. For this reason, they were directly glued on them to avoid moving samples from one holder to another one and prevent the samples composition from being affected by the glue.

This process worked correctly when the holders were used on the interferometer. However, when the samples were studied with the atomic microscopy they broke in small pieces. Different hypothesis were created to explain the reason why this problem appeared. The first option was that the samples received a little knock but when the problem appeared in new prepared samples this theory was eliminated. Then, the next hypothesis was that the glue created a reaction to the sample and therefore, they broke in small pieces.

To avoid this problem, the join process was done with silicone. In some cases, the samples were really small and the silicone covered the sample if it was not collocated in a really thin film. To do this film another holder was used. One drop of silicone was situated over the holder and with the other one it was spread over the surface. Then, the samples were situated in the middle of the holder. Using this new process, the samples could be analyzed with the atomic force microscope without any problem.

Then the samples had to be analyzed with a field emission microscope. To use this instrument, the sample should have a conductive or a semiconductive surface because otherwise, the surface would accumulate electrons and images could not be taken. In this case the samples were made from calcium oxalate and it is not a conductive material. Therefore, it had to be covered with a conductive material as gold or graphite.

The samples which were covered were the same ones that were analyzed with the atomic force microscope. Therefore, the samples were still joined to the metallic holders. The samples were covered with graphite using a machine which vaporized this material over the surfaces of the samples. Then, the samples were glued to a peg which is special to be located inside the field emission microscope.

5.2. Experimental Procedure

5.2.1. Materials

The material selected to do the experiments was calcium oxalate dihidrate. They were a generation donation of the fundació Puigvert. Concretely, as the experiments were based in the idea of analyzing kidney stones, the samples were from real patients who suffered urolithiasis. These samples had a percentage of calcium oxalate over 90%. This information was obtained from the analysis of Dr. Silvia Garcia from fundació Puigvert. She characterized the samples using an optical microscope and also an infrared spectrometry.

There were five different samples which had more or less the same composition. They were from five different patients. They were from different patients to verify the results. All the patients suffered hypercalciuria and that was the reason why they created the calculi. In the figure 5.1, it is possible to see the stones before beginning the experimental procedure. As it is possible to observe, the dimensions of this stones are not so big, however, they were painful for the patients.

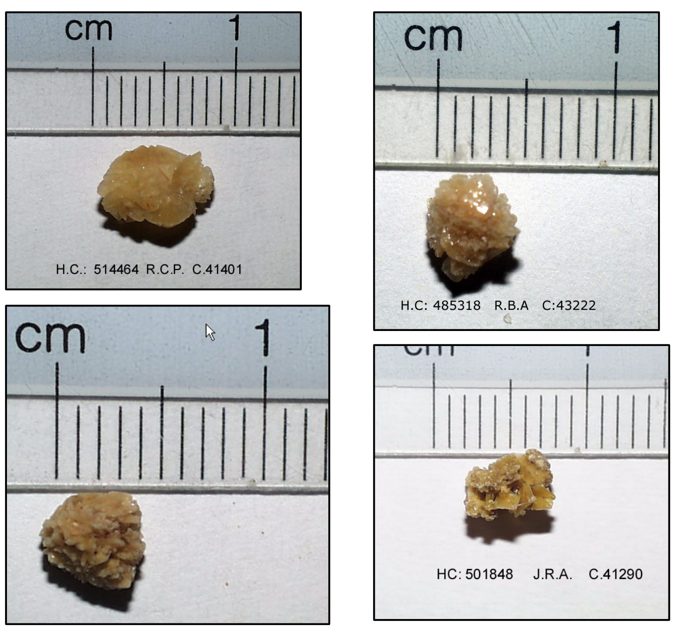


Fig. 5.1 Samples without any handling (A,B,C,D)

5.2.2. Optical microscope

The kidney stones were observed with the optical microscope to do a first contact and to identify the interesting surfaces. In that moment, it was observed that the samples were so big and irregular to be analyzed with the interferometer. Therefore, the samples were broken and the analyzing process was done again. Then, some pictures were taken which showed the crystals, these surfaces are the ones which shine in the following picture (Fig.5.2).

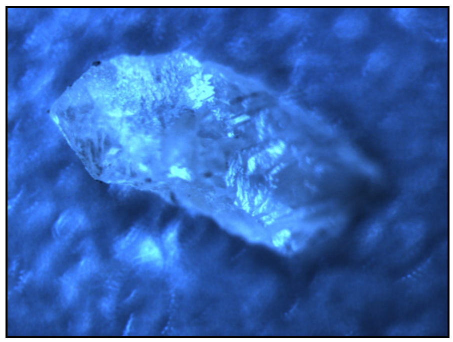


Fig. 5.2 Sample observed with the optical microscope.

Once they were located, some measurements were taken to indentify the dimensions of them. If they were too small, it would be impossible to take images from them with the atomic foce microscope (AFM) because they would be extremely difficult to find with the optical microscope of the AFM. Moreover, this process facilitated the posterior location of the study zones. In the following picture, it is possible see these zones and their dimensions.

This process was very important as the familiarization with the samples is vital to facilitate the following processes. After this preliminar analysis, the samples were glued on metallic holder samples in a way that the zones which wanted to be analysed were in the upper part. This holder were placed on the interferometer to begin a more intensive analysis.

5.2.3. Interferometric microscopy

An interferometer is an optical device which works with the interferences of light. This instrument divides a beam of light in two, one beam is reflected from the object which is analyzed and the other one is reflected from a reference mirror. Then, the two beams are recombined and the resulting interference phenomena are recorded in the interferogram. This information goes then to the Charge-Coupled Device (CCD) detector which, using a phase-mapping program which shows the topography of the sample. [31]

To begin the analysis with the interferometer, a quite large objective (X10) was used, as it was easier to find the surface observed in the optical microscope. In this process, it was important to place the sample always in the same position as the identified flat surfaces can be found easily in a next examination. Once the surface was found, the interferometer has to be focused through the use of z axis. This process was quite complex as the plate surfaces were not all located in the same direction and the samples were not very reflective. However, some surfaces were finded in each sample. When the crystals were found, the magnification of the interferometer was changed to X50 to see the surface more in detail. All this process was done using the vertical scanning interferometry (VSI) mode which is the most useful for this kind of samples because they had some large steps in the vertical axis. Then, the crystals had to be focused another time with the new magnification. When this process was finshed, the image was shown with some fringes which had to be minimised to obtain the best results. After that, it was possible to obtain some images and some values which described in detail different crystals of the sample. The following picuture shows the dimensions of the sample and also its inclination with respect to the plane.

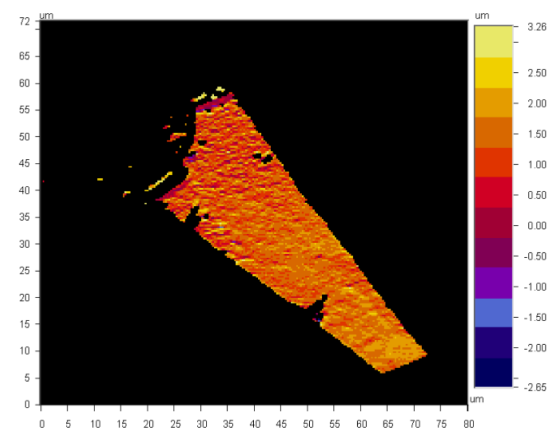


Fig. 5.3 image obtaiend from the interferometer.

Although the images directly obtained from the interferometer were useful, it was possible to obtain more information if an image processing software was used. For this reason, an intensive analysis of the crystals was done using a software of image processing. Using it, it was possible to define several paramenters and also to study the samples plate by plate.

The following images (Fig. 5.4) show the topography. There are two horizontal cuts and one vertical cut consecutively.

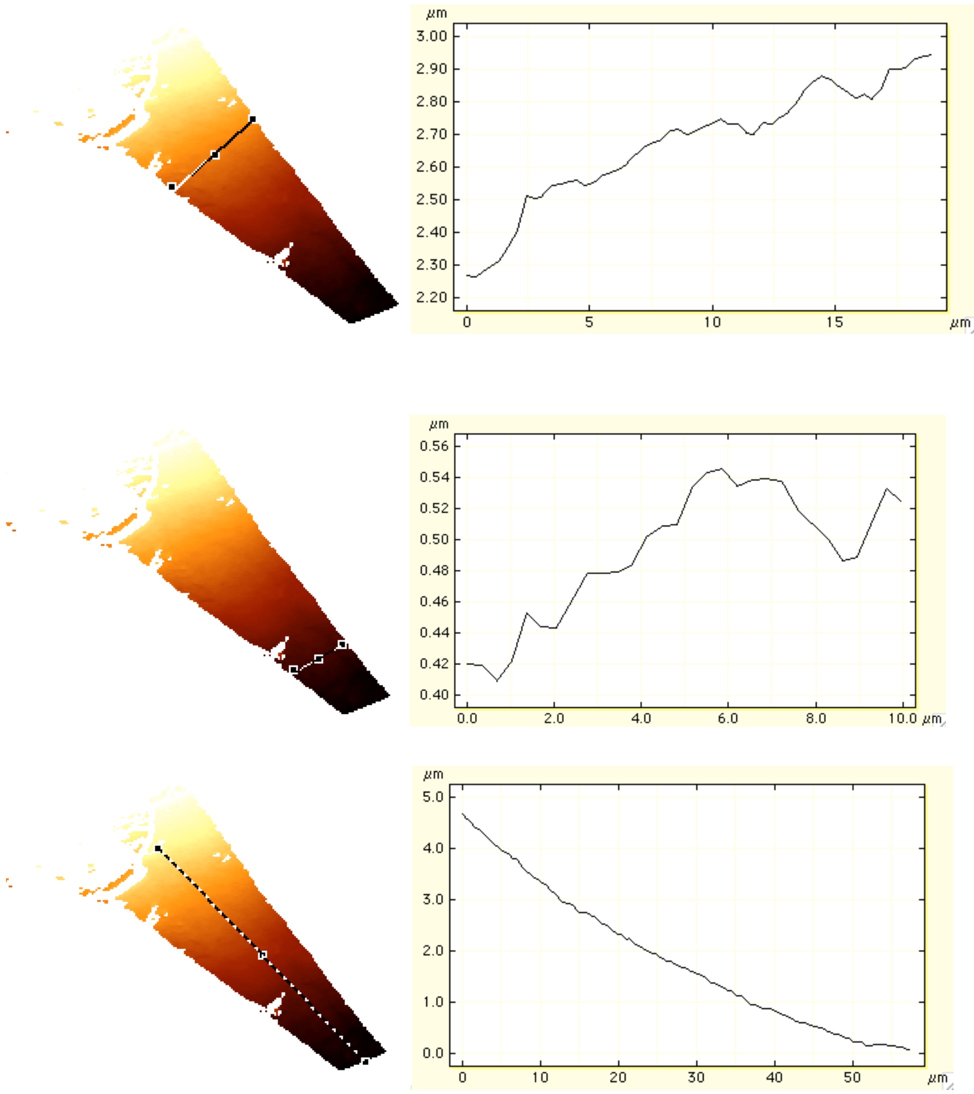


Fig. 5.4 Analysis of the cristals

Moreover, it is possible to do an histogram of a selected part and also to analyse the surface profile of the same section. The following picture shows the section chosen, it is one of the flatest zone of the crystal.

All this study was done to located, studied and define flat zones which will be analysed with the atomic force microscope. As if this surfaces are not so flat they can not be useful from the next technique.

5.2.4. Atomic force microscopy

An atomic force microscope (AFM) is an instrument which can take images and measures of surfaces on a fine scale, down to the level of molecules and groups of atoms.

The basic idea of how an atomic force microscope works is that a sharp tip, which is located at the end of a flexible cantilever, is moved across the surface sample maintaining a constant force. When the tip moves in proximity to the investigated object, forces of interaction between the tip and the surface influence the movement of the cantilever.

There are different kinds of tips, depending of which kind of surface wants to be analyzed. Their radius can vary from 2nm until 20nm. Also, there is a laser which is projected over the cantilever and then is reflected to a photodiode detector. This process is used to monitor the interaction between the tip and the sample observing the variation of the laser. Finally, there is a piezoelectric tube scanner which conducts the scanning motion.

The area which can be scanned can vary considerably but the instrument used to do this experiment scans ranges up to 120 microns on the X–Y axes, and has a Z range up to 6 microns. Moreover, it has a high-resolution scanner (0.5 microns X–Y axes and submicron Z range). [32]

The process of the analysis with the AFM is really complex due to the lack of large flat surfaces. Their orientation was another problem because they are not parallel to the cantilever and in some cases the tip cannot analyze the complete surface.

The process begins with a first touch in contact with the microscope as its operation is difficult. The first analyses were done with holders which only contained silicone. These studies were done to be familiarized with the instrument and also to see the aspect of the silicone. It was important to be able to differentiate the aspect of silicone from the sample in the case that some samples were covered with the silicone accidentally. After some studies of this material, it was possible to see clearly it aspect and also to control easily the instrument.

Then, the analysis of the real samples began. The first sample which was analyzed was the named A in section of materials. This sample was considerably big and hence it was possible to create different holders with samples.

These samples have quite big and flat crystals as they can be seen with the eye tie. However, as they were the first ones analyzed, some problems to localize the surfaces appeared but in a few days some good images were obtained. At that moment, some strange surface appeared in the images. There were spherical structures which were spread throughout the whole image. That phenomenon was very surprising and worrying. Therefore, different hypothesis were established to discover what were that surfaces. The following image shows the spherical surfaces.

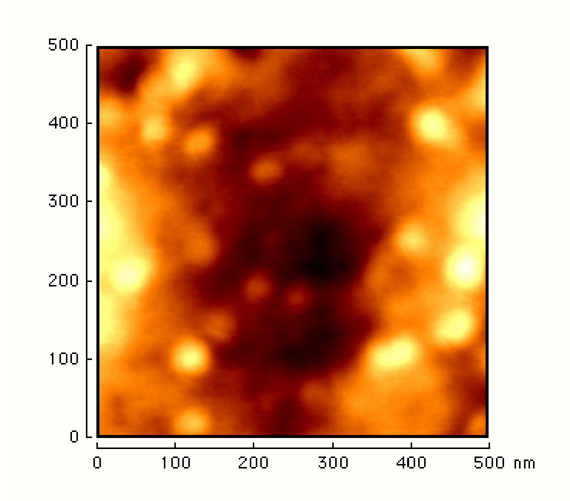


Fig.5.5 AFM image (dynamic mode) of the distribution of the identified spherical nanoparticles

The first step which was done to solve the doubt was to analyze another holder of the same sample to see if this phenomenon appeared in other parts or if it was a localized problem. When the second sample was analyzed, it was possible to observe that the same kind of structures appear again. However, the distribution of them was quiet different. Their distributin was very similar to the calcium phosphate (apatite)-covered organic particles (nanobacteria) obtained by G. Aloisi (2008). At that moment, the study of the kidney stones did a big change as it was possible that some important structures were found in the analyzed samples.

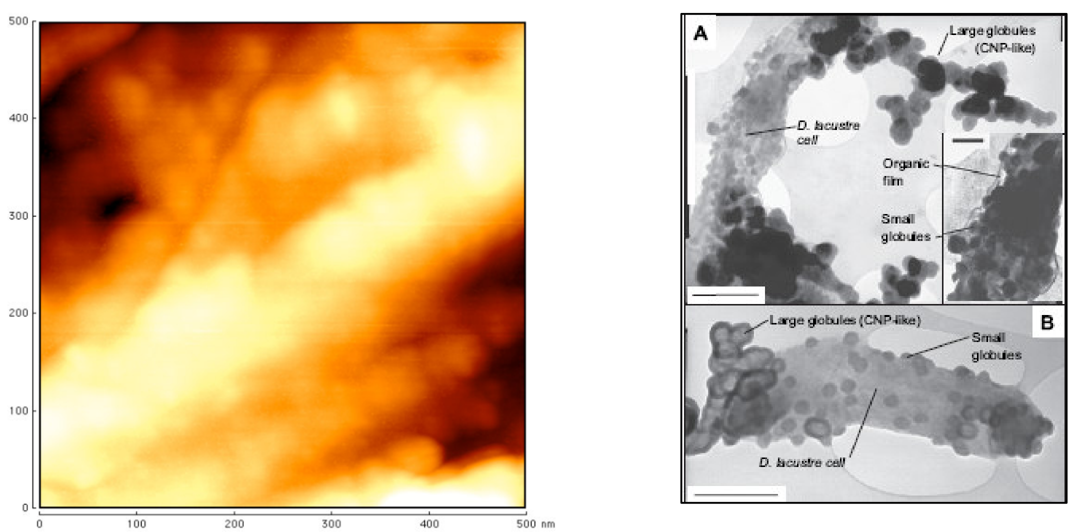


Fig. 5.6 Comparison between observed nanoparticles (left, AFM this work; right, TEM Aloisi, 2008)

The next step was to dimension the spheres to try to define which kind of structures they were. To do it, an image processing software was used again. That program has a command which can create a diagram in the x and y direction of a surfaces. Therefore, it is possible to see the length and the height of the structures. The figure 5.7 shows an example of this kind of diagram. The problem was to define a way in which all the structures were analyzed with the same criteria.

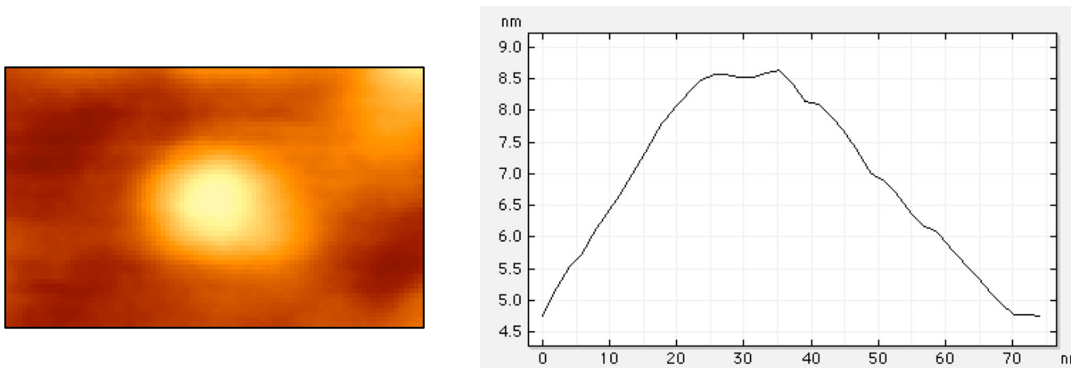


Fig. 5.7 Diagram of one of the spheres

It was found that the best command to study the different surfaces was “particle analysis”. That option isolated the structures from the flat surfaces. Therefore, all the particles could be analyzed using the same criteria because the diagram could begin and finish in the position where there was a particle. The other surfaces, which could deform the result of the analysis, were omitted. The following picture shows the difference between the complete image and the image after the process of particle analysis. However, to do this process, two other processes had to be previously done. Firstly, the command “density slice” had to be selected. This command shows the surfaces with the same density. Using this command, it is possible to see more or less the final result of the particle analysis. Then, the command “threshold” had to be selected. Without this, the process of particle analysis did not work properly.

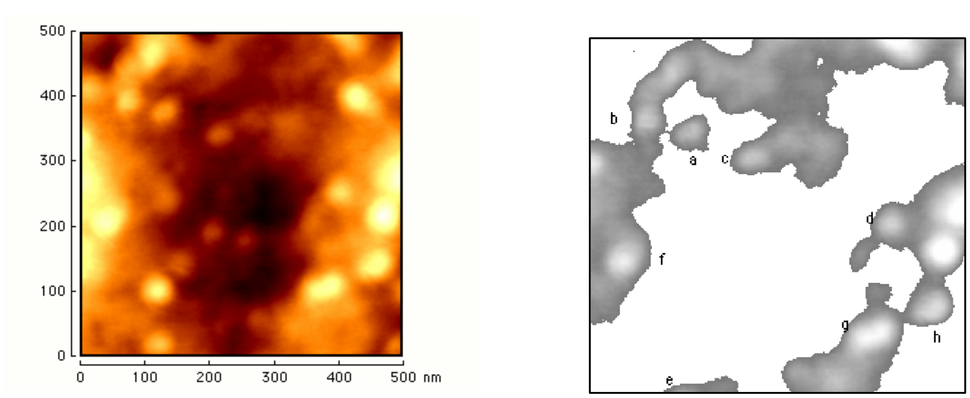


Fig.5.8 mage before and after the process of examination

When the particle analysis was done, it was possible to begin the analysis of these structures. To do these studies, some hypotheses were created. Firstly, the shape of these particles had to be defined. The first suspicion was that the structures were spheres. Using this first idea, a new problem appeared: it was unclear whether the sphere was situated over, under or in the middle of the base surface. As the tip of the atomic force microscope cannot touch the base of the sphere, they can be over the surface and the image only shows a curve. This supposition was rapidly discarded because showing the curves obtained from the study of different particles, the sphere that could fit into the curve are bigger than the distances between the higher part of the curve and the artificial floor. Therefore, the option that a part of the sphere was over the surface and the rest was below seems the most consolidated. Both options are explained graphically in the following scheme.

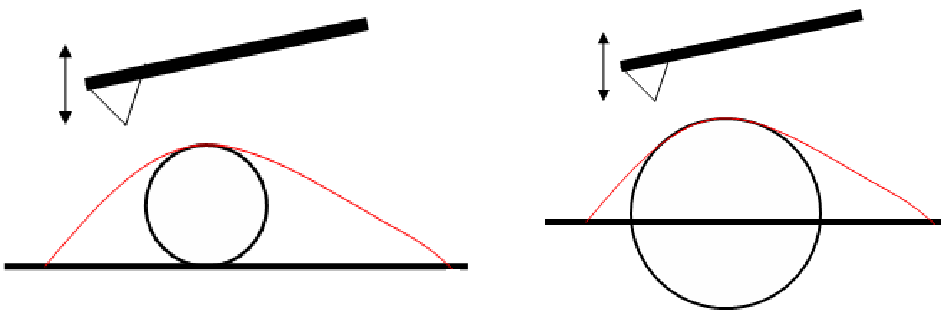


Fig. 5.9 Scheme of the situation of the spheres

Using this new hypothesis as the base of the analysis, a new problem appeared. To compare the different spheres, the diameter of them had to be defined. As part of the body of the sphere was under the surface some trigonometry had to be used. The only information available from this structure was the curve obtained from the atomic force microscope. First of all, the highest point of the curve had to be found. Then, another parameter which was easily obtained was the inflection point of the curve. When, these three points were defined, the distance between them could be measured. With this distance, it was possible to draw a triangle like in the following scheme.

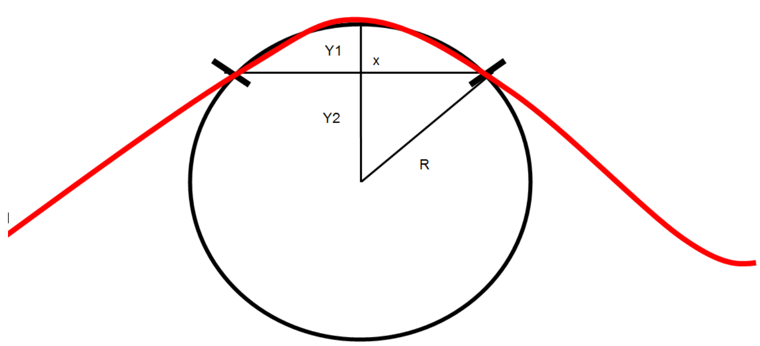


Fig. 5.10 Scheme of the mathematical process

Using the Pythagoras theorem, it was possible to define the following equation:

$$R^2 = x^2 + y_2{}^2 \quad \textbf{(5.1)}$$

Also, it was possible to define that:

$$R = y_1 + y_2 \quad \textbf{(5.2)}$$

Mixing both equations, it was possible to calculate the value of the radium of the spheres:

$$R = \frac{x^2 + y_1{}^2}{2y_1} \quad \textbf{(5.3)}$$

When the mathematical process was defined, a way to do it had to be found. To open the files with the information of the curves a mathematical software was used. Using it, it was possible to see the values of the x and y axis in different columns. Then this information can be imported to another software. The first step was to create two vectors with the information of the x and the y axis. The next process was to create a fitted curve with these two vectors. To do it, the first step is to find the kind of equation that defined with more precision the curve showed in the image processing software. In this case, the best option was to select a third grade equation. Then, the fit command had to be used to create the values of the four variables of a third grade equation. After this process, the curve was completely defined and the inflection point and the higher point could be found. To obtain these values the following equations were necessary.

The following equation was the base for the rest:

$$f(x) = ax^3 + bx^2 + cx + d \quad \textbf{(5.4)}$$

To find the inflection point, the second derivative had to be done and had to be equaled to zero.

$$\text{Inflection point}=\frac{\partial^2 f(x)}{\partial x^2}=0 \tag{5.5}$$

$$\text{Inflection point}=\frac{-2b}{6a} \tag{5.6}$$

On the other hand, to do the maximum height a similar process had to be followed. The base equation had to be derived and equaled to zero.

$$\text{Maximum height}=\frac{\partial f(x)}{\partial x}=0 \tag{5.7}$$

$$\text{Maximum height}=\frac{-\frac{1}{3}\left(b\pm\left(b^2-3ac\right)^{\frac{1}{2}}\right)}{a} \tag{5.8}$$

When this process was finished, the value of the different diameters of the sphere was obtained. In that moment, it was possible to compare different diameters of the same sphere to define if they were real spheres or they were other kind of structures. Comparing various diameters in various cases, the result was that the hypothesis describing them as spheres is true as the difference between them was never higher than 10nm. Therefore, it was assumed that the structures were spheres. Moreover, the two hypotheses that were proposed previously were verified.

When the structures were clearly defined, the next step was to analyze the other samples, those are the named as B, C and D in the section 5.3.1. The fifth one was not used because it was too small and it could not be used in more experiments to verify the results. For this reason, it was discarded.

The samples were broken as in the previous case and they were joined directly with silicone to the holders. Then, they were analyzed with the atomic force microscope using the same process as the previous one. Then, some images of each sample could be taken. Even though, some samples had to be created newly, because, the crystals that they had, were not enough flat or enough big to be analyzed. Finally, some images of each sample with different scanning zones where taken and they could be analyzed.

5.2.5. Field emission microscope

After, the study of the samples with the AFM another technique was used to study the spherical structures obtained with it. This technique was the field emission scanning electron microscopy (FESEM). It is an analytical technique which is commonly used to study the molecular surface structures and their electronic properties. It is a kind of scanning electron microscopy. It consist in apply a high negative voltage in a metallic tip which is located in small distance from a screen which had a fluorescent coating. The tip produces electrons by field emission. The field emitted electrons travel along the field lines and producing bright and dark patches on the fluorescent screen related to the individual exposed planes of atoms. The reason why it was used this technique and it was not used a common scanning electron microscope is because it produces clearer, less electrostatically distorted images with spatial resolution better than conventional SEM. [33]

To use this technique the samples were vaporized with carbon as it was the only was that this kind of samples could work with this microscope. That means that these samples cannot be used newly in any other studies.

The first step was to put all the samples in the sample peg to be introduced at the same time inside the FESEM. It was important because analyzing all the samples together, the vacuum process only have to be done one time. This process takes a few minutes; therefore, doing in that way, it was avoided losses of time.

When, the samples were inside the microscope had to be focused the initial point which was defined previously. When, it was defined it was possible to know the order of the samples.

The process of analysis begins with the studied of the all samples to do a first contact with the technique. Then, the magnification of the image was increased to observe some independent crystals of the calculi. Through, this process some problems with the astigmatism appeared at the time of focus the microscope. However, they could be solved re-focus the microscope. In the following images, it was possible to observe the different calculi.

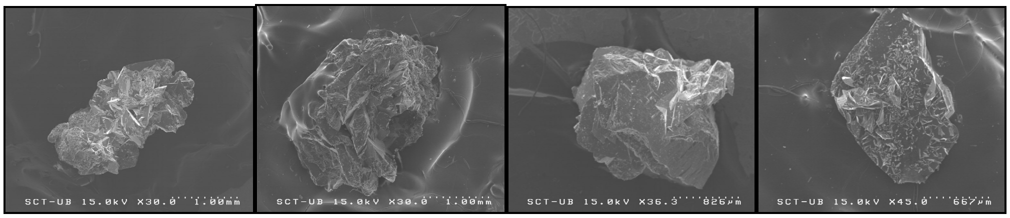


Fig. 5.11 FESEM images of the four samples (A,B,C,D)

Finally, the last examination was done with a very high magnification. In the previous study of the crystals, all the surfaces of them looked like flat. The idea to do this last increase of magnification was to try to see if it was possible to define the spherical structures which were observed previously. When, the scale of analysis was round 1-1.20um, it was possible to observe the spherical surfaces.

The information obtained with this technique is qualitative. As the images had not more information the simple picture and it is not possible to study the images with image processing software. Therefore, these images only can offer results from the image and not from the study of it.

6. Results and discussion

6.1. Results of characterization

The characterization process was done using interferometery. Through this technique, it was not possible to define the chemical composition of the kidneys stones. However, the doctor Silvia Garcia did a previous characterization to define which composition had each calculus.

The characterization did in this project was related with the topography of the stone. It was to define which kind of surfaces had the different samples and also the dimensions and the inclination that they had. The reason why they were so important was because to be analyzed with the atomic force microscope, they must have a minimum dimension to not exceed the lateral resolution of the microscope. Besides, the inclination was analyzed for the same reason as the atomic microscope also has some limitations in the z axis.

Observing the following graphs which were obtained from different crystals of the different samples was shown that in any case the maximum inclination was not over 6um which is the maximum which can be support from the atomic force microscope. Normally, the inclination of the different crystals was around the 1 um.

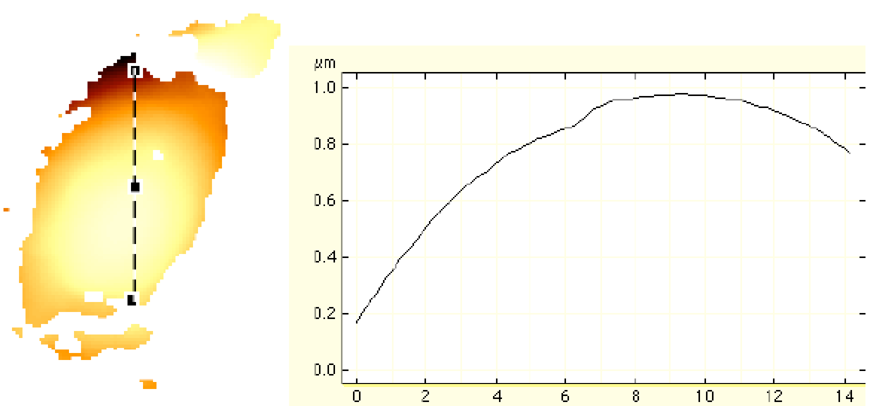


Fig. 6.1a Analysis of the topography of the crystals obtained with the interferometer

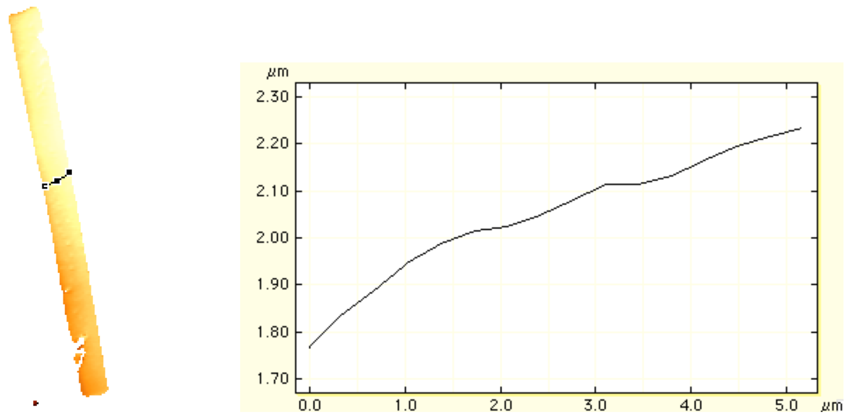


Fig. 6.1b Analysis of the topography of the crystals obtained with the interferometer

The dimension of the crystals can vary considerably from one to the other. As it is possible to see in the following images some of them are independent crystals and another are zones with some surfaces which reflected. However, in all the reflected zones, there were crystals which were bigger than 1um. The regions, which will be analyzed with the atomic force microscope, will have approximately this dimension. Moreover, if there are some regions which are bigger, they will be easily found with the AFM.

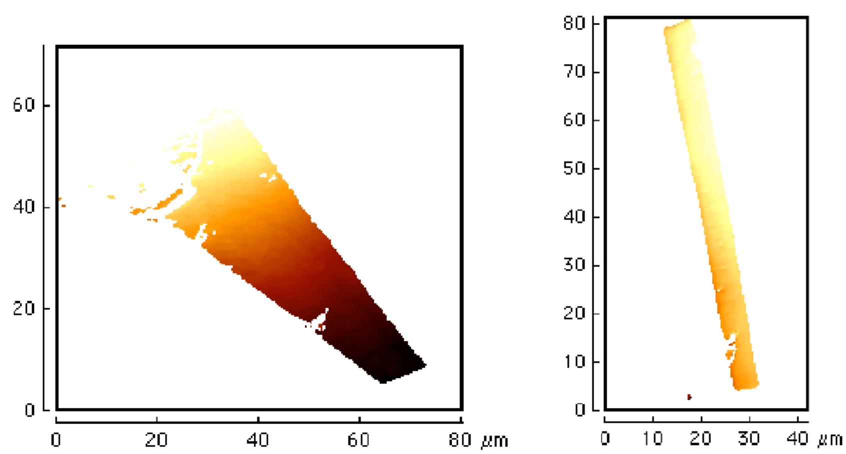


Fig. 6.2 Dimensions of the images obtained with the interferementer

On the other hand, it is possible to observe that some crystals are not complete. That means that some regions of the same surface were not a reflective zone that means that these regions were not enough flat, and also, they were not useful for the atomic force microscope.

6.2. Results of atomic force microscope

There were four different samples which were analyzed with the atomic force microscope. The first one (A) was analyzed in two different sections of the same samples.

In the first image it is possible to observe that there are some independent CNP-like particles. Some of them are more close to surface of the stone and some others are located more introduced in it. The second image shows aggregations of CNP-like particles.

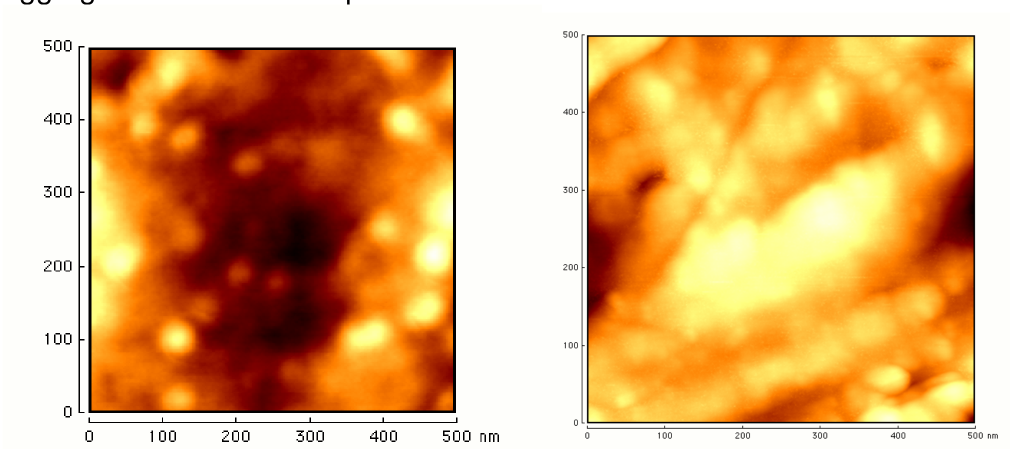


Fig.6.3 AFM images of sample A

The following image show one of the clearest images of the CNP-like particles. This sample had different flat surfaces, in one of them was obtained this image where is possible to see clearly the aggregations of particles. However, it is also possible to differentiate the rounded structure of each independent particle.

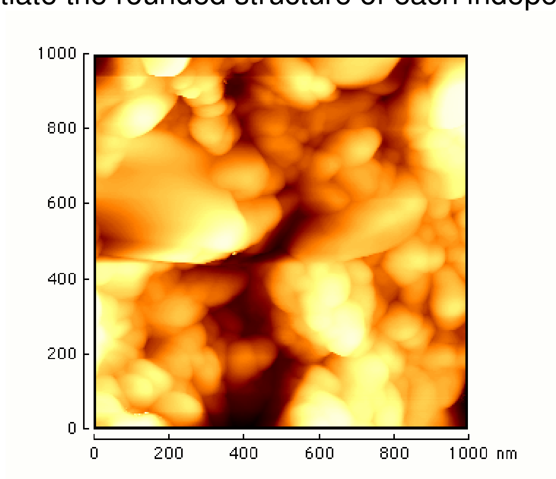


Fig.6.4 AFM images of sample B

The following sample studied had a very sandy aspect, therefore, there were few flat surfaces where could be obtained images. On the other hand, it was extracted the following images where clearly are aggregations of particles. In the first figure, the aggregations have a rounded aspect and it is not easy to define if they are one or several CNP-like particles in each group of aggregations. Besides, in the second image the same problem appear another time. There is only possible to observe particles clearly only in the central zone.

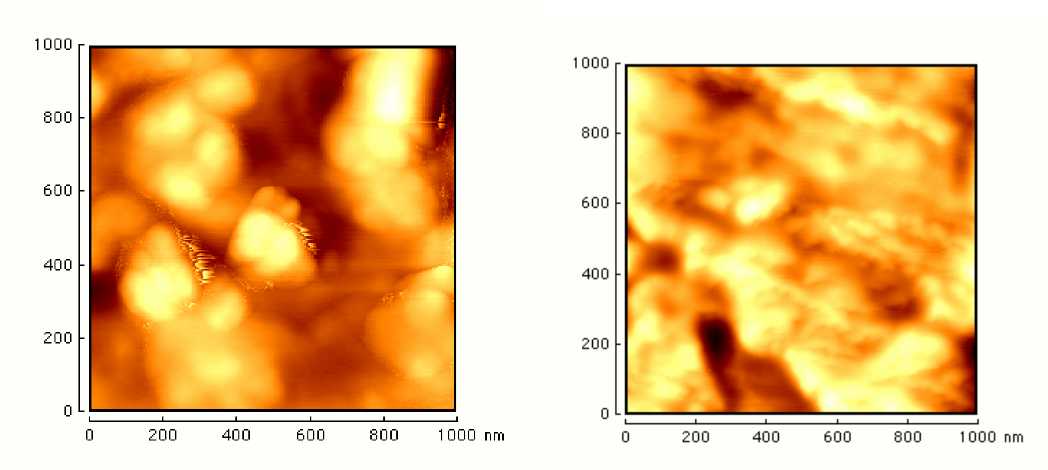


Fig.6.5 AFM images of sample C

Finally, the last sample analyzed had two different aspect clearly differentiated. The first image shows a huge aggregation where is especially difficult to define the particles one by one. The whole image is full of aggregations. However, in the second image the CNP-like particles are clearly independent. Therefore, there are little aggregations, the particles are clearly defined, though, they are in groups.

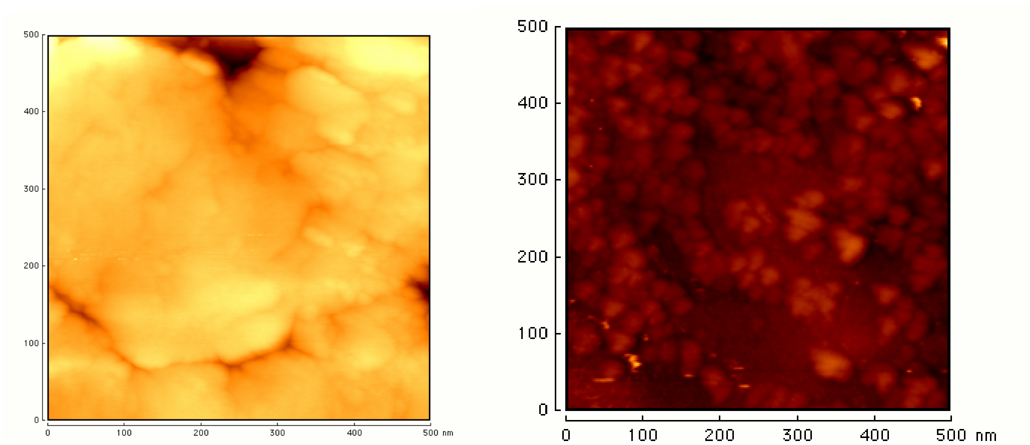


Fig. 6.6 AFM images of sample D

All this images will be analyzed studying particle by particle using the mathematical procedure explained in the previous section.

6.3. Results of field emission microscope

This technique helps to verify the results obtained with the atomic force microscope. All the samples which were used in the other technique were covered with carbon to realize this kind of studies. Therefore, they cannot be used newly in other techniques. The results obtained from this technique can not be analyzed as they only gave images of the samples without any other information. Therefore, the results are quantitative not qualitative.

When all the samples were inside the field emission microscope the study begins with and analysis of the whole sample to identify which zones were more interesting and which ones had to be discarded. The crystalline zones were selected as they were ones which were studied with the atomic force microscope.

Then, the magnification was increased and the study was done with hundreds of microns. The surfaces of the crystals looked like as flat surfaces and there were not any presence of the CNP-like structures. This happened in all the samples and it is possible to observe in the following picture, which is obtained from sample A.

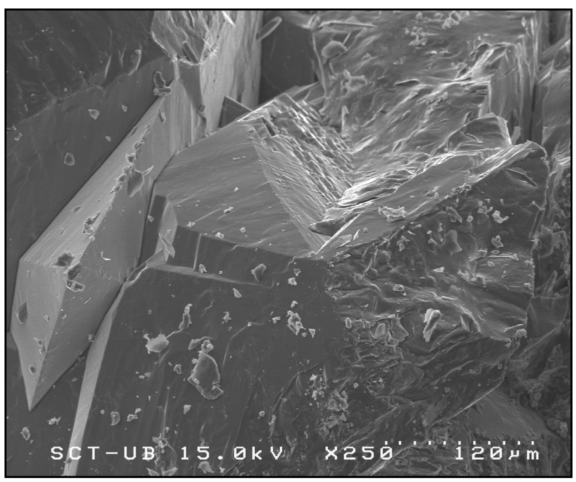
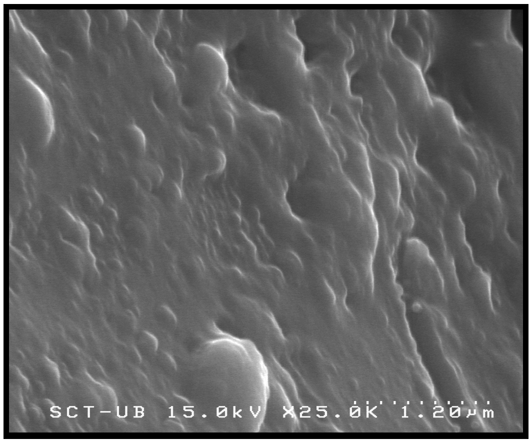


Fig.6.7 Field emission image of crystals of a calculus (sample A)

When the magnification was increased to some microns, it appeared CNP-like particles in the surfaces of the crystals. The dimensions of these particles were really close to the ones obtained with the atomic force microscope. The information of this images as it was explained previously is quantitative, therefore, the dimensioning of this structures is not really precise. However, they were around one separation which means that they measured approximately 120nm. On the other hand, it have to be taken into account that this surfaces has some angle in respect to the z plane which mean that it is possible to have a little bite of an optical effect which enlarge the structures. Using this presumption the results are more close to the once obtained with the atomic force microscope. In the following pictures, it is possible to see independent CNP-like particles and also some zones where they were located creating a structure which look like aggregations.



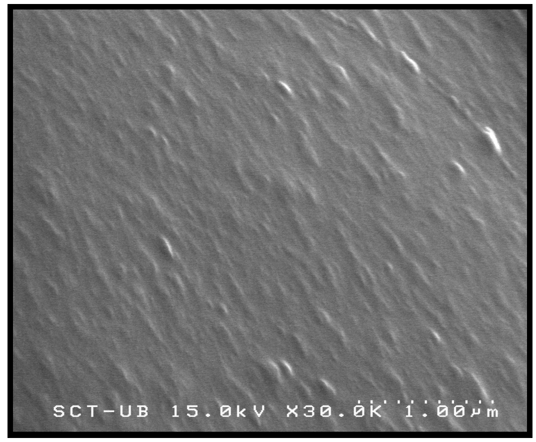


Fig. 6.8 Detail of the CNP-like particles with the FESEM (sample B and D)

Using the information obtained from the images of the field emission microscope, it is possible to assume that the particles obtained from atomic force microscope and these ones are the same.

6.4. Dimension of analyzed structures

In order to define the dimensions of the CNP-like particles which were in the images of the atomic force microscope was used the procedure explained in the section methodology.

Through all this process, there were obtained the diameter of 76 different CNP-like particles from the 4 different samples. In the following graph, it is possible to see the diameters of the different particles from the smallest one to the biggest one. Studying it in detail, it is observed that there is a continued growing rate during the samples until they arrive to 200nm of diameter. In that moment, the growing increases suddenly and the diameter of the particles is considerably higher.

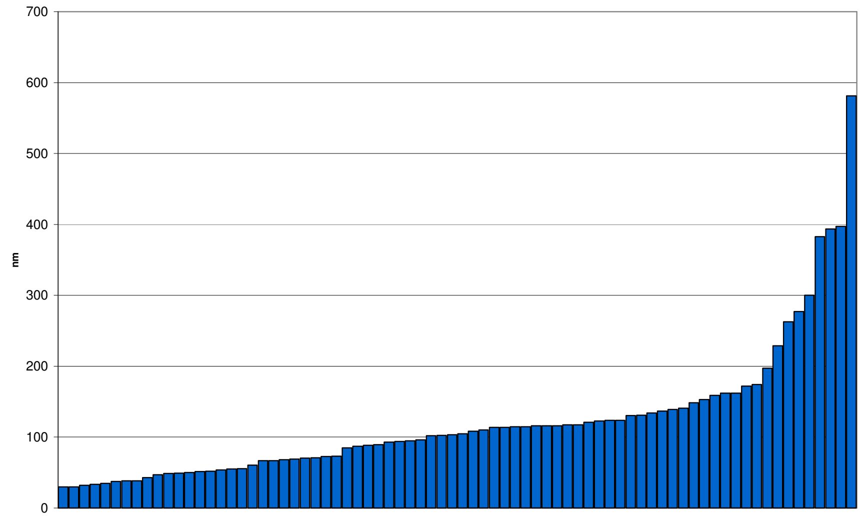


Fig.6.9 Distribution of the diameters from the smallest to the biggest one

Observing this suddenly change in the growing rate, it was possible to define that there are 7 CNP-like particles which are considerably larger than the previous once as their diameters are higher than the others. Observing the CNP-like particles separating them sample by sample, it appears another common point between these 7 CNP-like particles. All of them where from the same sample (Fig.7.4.2)

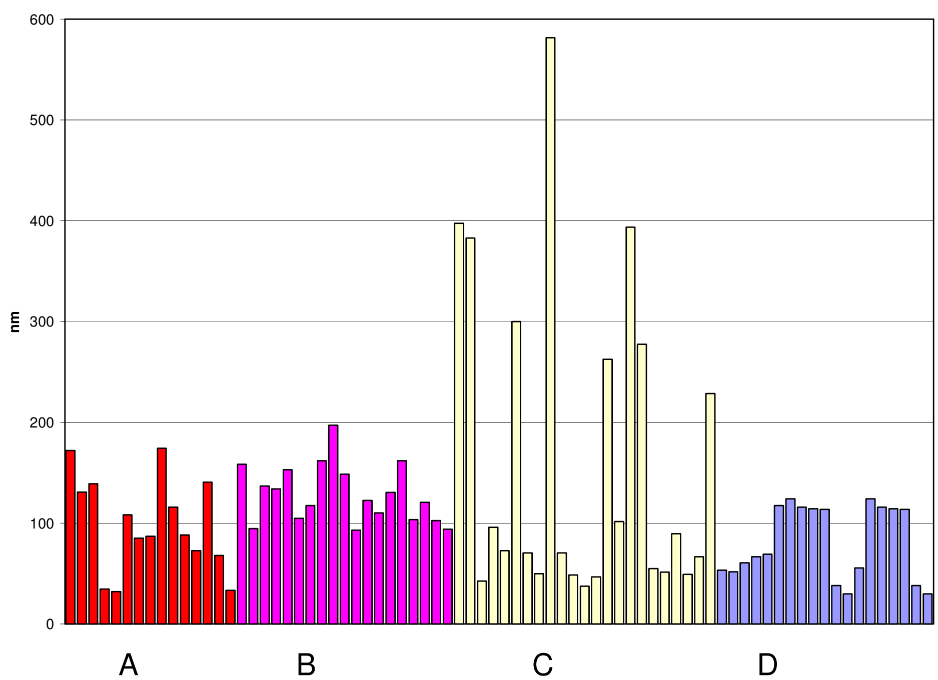


Fig. 6.10 Diameter of CNP-like particle sample by sample

Furthermore, if the region of the third samples is analyzed is possible to observe clearly that there are two different patterns which are clearly defined. The first one is the picks which once where exposed in the previous paragraph and the other part are the CNP-like particles which have the same range of diameters of the others. Therefore, the first idea was that some problem appeared in the moment that the information was taken to do the calculation. However, studying the topography of this sample (C) another time (Fig.6.5) was possible to observe that this sample was the one which had the highest number of CNP-like particles groups. Therefore, the strange results can be defined because the analysis instead of being done over independent CNP-like particles, it was done over group of some CNP-like particles which had a rounded aspect. Consequently, these values do not have a significant result for this final analysis.

When this suddenly variation of the growing rate was explained, the next step is to analysis the CNP-like particles instead of separating them sample by sample for percentages of their diameter dimension. In the following graph it is possible to see this distribution. This graph has several important results. As it was defined previously, the results of the suddenly growing rate were discharged because they could deform the analysis of the results. Therefore, the following graph only takes into consideration the samples with a diameter smaller of 220nm.

Furthermore, there are not any values under the diameter of 30 nm which is a relevant result for this study and also verify the results. Another important part to study is that the approximately the 20% of the samples had a diameter between 100-120nm. This value can be associated qualitatively with the results obtained with the FESEM which verify the results of this analysis. Besides, the other particles are distributed in the other ranges (20-140nm) more or less uniformly as they are between the 10-13% in all the sections.

On the other hand, the range groups of 140-200nm are smaller, thought, they involve approximately the 12% of the samples which is not a rejectable result.

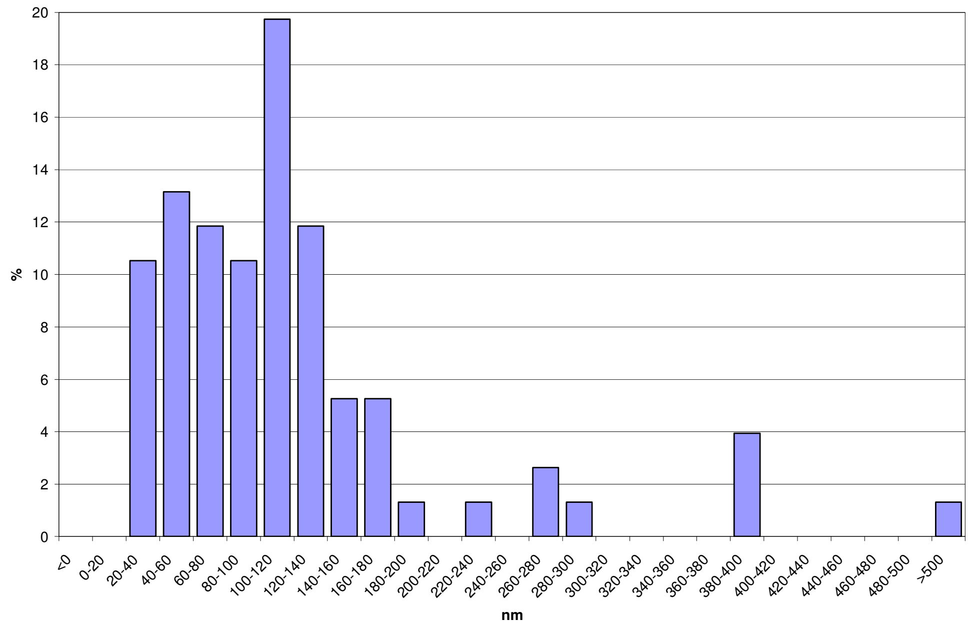


Fig.6.11 distribution of the diameters of the CNP-like particles

6.5. Discussion of the CNP-like particles comparing with previous studies

The study of nanobacteria was focused from different points of view the last decade. These different opinions provoked that this topic is involved in a big controversy because it is not defined if they are living organisms, or what is their origin, or how do they mineralize. Some of the most important reason because this question is not answered is because their nucleic acid is not sequenced yet.

The results obtained from this study can be compared in the different theories that are created around the CNP-like particles. First of all, it is important to observe during this project that all the samples analyzed had CNP-like particles. They had different aspects and size depending on which sample was studied but they appeared in different zones of all of them. This observation can be compared with the results of N. Çiftçioglu et al. which affirm that the 97,2% of their samples contained nanobacteria. Moreover, this theory is supported for the different scientist because as it was defined by F. Grases et al. the 90% of calcium oxalate calculi contained small amounts of phosphates in their core. Therefore, it was not strange that all the samples had CNP-like particles.[23]

Another relevant result is the size of the CNP-like particles. Their diameter oscillated between 30-200nm. G. Aloisi defined the size of the CNP-like particles between 50 to 200nm. EO. Kanjander and N. Çiftçioglu defined their diameter between 80 and 500nm. Besides, J. Materl et al agree with this definition. In both cases, the minimum diameter of the CNP-like particles is bigger than the one obtained with the examination with the atomic force microscope. The reason why this happens can be explained because the resolution of this technique which is better than once used for the other scientist. They use scanning electron microscope which is also a really potent microscope but it has a lower resolution than the AFM. Moreover, the maximum diameter of EO. Kanjander and N. Çiftçioglucan be defined ignoring the possibility to study an aggregation of CNP-like particles instead of independent CNP-like particles. The reason why they considered them as an independent CNP-like particle can be for the same reason as in the previous case for a low resolution of the microscope. [25,28,29]

The next comparison is related with the aggregations of CNP-like particles that there were in the images obtained from the AFM and also from the FESEM. In both cases, it is possible to observe that there are some particles which are isolated but also there were some ones which were located close ones to the other creating a group of them which look like a colony. The creation of colonies was one of the fundamental points for all scientists which are involved with these investigations. Bacteria have the ability to create colonies. However, G. Aloisi explains that the end product of the bacterial miniralization process also have the ability to create colonies. Furthermore, J. Martel et al. explain that the CaCO3 precipitates create aggregations in the form of colonies. [29]

Furthermore, the images obtained from the AFM and the FESEM are similar to the ones obtained from G.Aloisi and J.A. Jones et al. The reasons why it is possible to do this affirmation is because the organization of the CNP-like particles is similar and also the dimensions of them. In the following pictures is possible to observe different images from the other scientist, thought, the microscopy used by them was different than the used in this project. [27,28]

The first image is from J.A. Jones, it is obtained from a SEM. This image has an aspect similar to the ones obtained using the FESEM (Fig. 6.12)

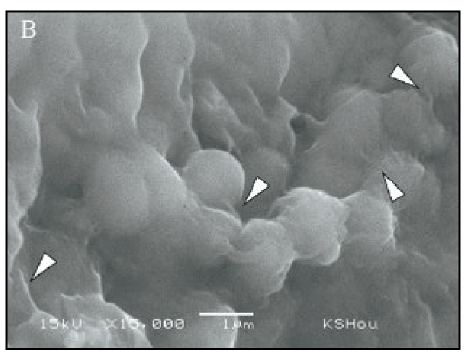


Fig.6.12 SEM image reveling CNP-like small spherical apatite units of a calculus (J.A.Jones, 2008).

The next image is from G.Aloisi. It shows the suplahte- reducing bacterium D. lacustre forms CNP-like particles when immersed in a supersaturated cultive. The structure of the aggregations is similar to the ones were showed previously in the images obtained with the AFM. [28]

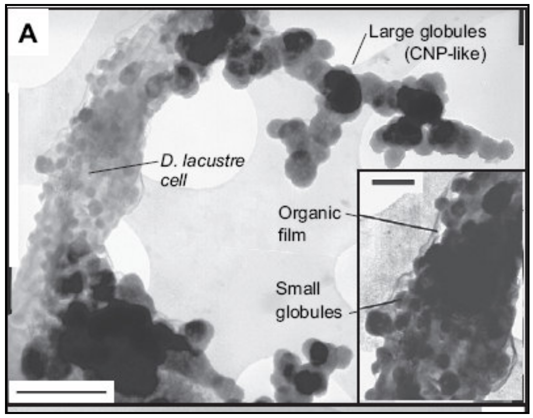


Fig. 6.13 CNP-like particles [28]

Finally, the last point which is important to compare is that this kidney stones are from Spanish patients and in the other studies the origin of the samples where form Finnish and American patients.

7. Mars reference mission

The concept to create a Mars reference mission was from NASA concretely the NASA Mars Exploration Team at the Johnson Space Center (JSC) in the 1997. Inside this team, there were personnel who represented several NASA field. The main idea of this project was to develop different essential aspect to realize the first manned mission to Mars such as the life support systems, the environmental protection or the propulsion. These aspects and more other were studied from different points of view to increase their effectiveness, reduce risks, and reduce cost.

During the following years, NASA created various addendums which improved the contents of this project. For example, in 1998, they created an addendum which had some changes which were manifested in variations of the strategy and the development of the mission. Moreover, in 2001 created another which was more related with the Mars surface. [34]

7.1. Other proposal for Mars manned exploration

There have been proposals and projects in relation of this topic since the 50s and they were done all over the world. All the different space agencies and some particular groups created projects in relation with missions to Mars. However, they focused from different points of view.
Wernher von Braun was the first person to make a detailed technical study of a Mars mission. His work was published in his book *Das Marsprojekt* (1952). However, it did not be translated to English until 1962.

In the same year, Aeronutronic Ford, General Dynamics and the Lockheed Missiles and Space Company made studies of Mars mission designs as part of NASA Marshall Spaceflight Center "Project EMPIRE". However, this project was only studies not a defined proposal.

During 60s, the Soviet Union did two different proposals to do a manned mission to Mars. They were the Martian Piloted Complex (MPK) and the *Mars Expeditionary Complex* (MEK).

During 80s and 90s a series of conferences named The Case for Mars were held at the University of Colorado, they tried to define new concepts which will help to realize mission to Mars. In 1989, NASA design a new proposal named 90 days study which was based in the idea to establish a permanent base in the Moon and then they created the mission to Mars. In 90s, it appeared the Mars Direct concept created by Zubrin which was the first one to define the idea to use in situ resource utilization to manufacture propellant from the Martian atmosphere. Then, it was done the NASA Reference Mission and their addendums.

In 2004, it began the first European project which was the AURORA. This program had the long-term vision of sending a human mission to Mars by 2030. Nowadays, The Russian space agency, ESA and NASA are working in different plans and proposals to created new strategies to a possible Mars mission. [35]

7.2. Characteristics to human risk in Mars reference mission

The Mars reference mission v1 was not really accurate with the problems involved with health of astronauts. It only took into account two main factors which can be a risk for human health: the radiation and the problems related with long periods in microgravity. However, they did not realize a deep analysis of all the sickness and the risk that human life could find during a mission to Mars.[34]

On the other hand, the addendum did in 2001 which is related with the surface exploration of Mars, is more precise in this topic. It takes into account that if the astronauts have to spend 18 mounts on the trip they will need some medical care. It will be typically involved with routine activities but it will have the capacity to handle more serious situations. This addendum defines the more possible sickness that can appear during this kind of mission. It considers the decompression sickness, the radiation sickness, bone fractures, traumas, desconditionings, depression and stress, infections and toxic exposures. Apart from analyzing the more problematic sicknesses, they defined a really important idea which is “the most effective and least expensive method of delivering medical care is through the prevention”. [35]

7.3. Urolithiasis involved with future Mars missions

The Mars Reference Mission takes into account radiation and effect of microgravity in the bone degradation but it does not mention the urolithiasis problematic. It is demonstrated that the astronauts have more kidneys stones onboard than on Earth. In the cases recorded, the astronauts suffered urolithiasis in the post-flight period. The problem of a mission to Mars will appear if they suffer urolithiasis once the astronauts are to Mars, as the medical equipment for this kind of mission will be not prepared to react to this kind of situation. Therefore, as the addendum of surface exploration proposed the prevention of this illness is extremely important because the possibility to fast-return from Mars is not an option in these kind missions.

To include this problematic in the Mars Reference Mission has to be analyzed in detail different aspects of the mission. The first aspect which has to be studied is the time that the crew will spend in microgravity. To do this analysis it has to been taken into account the different trajectories that can be selected to do this mission. Trajectory options between Earth and Mars are generally characterized by the length of time spent in the Mars system and the total round-trip mission time. In the following pictures is possible to observe the three mission profiles that are studied to realize the mission. They are the short-stay, long-stay and fast transit mission profile consecutively. [34]

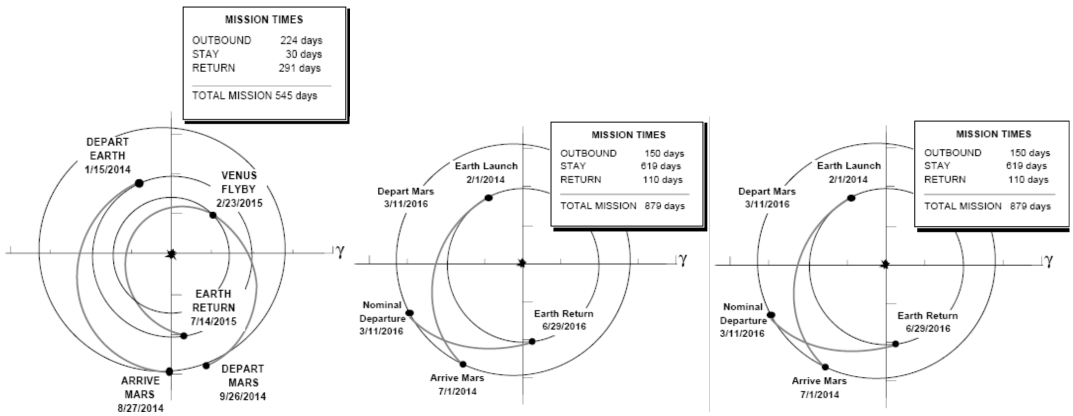


Fig.7.1 Mars reference mission profiles (S.J. Hoffman, 1997)

The goals and objectives of the Reference Mission focus on allowing human crew spend the greatest amount of time on the surface of Mars for the investment made to transport them there and to learn as much as possible how humans react in this environment. Therefore, the short-stay mission profile is clearly discarded because the stay on the Martian surface is only between 30 to 90 days. Considering the other two option, the long-stay trajectory option was considered to be best as it is able to satisfy the greatest number of mission goals and objectives. This kind of mission has approximately 450 days on board under conditions of microgravity. Therefore, when the crew will arrived to Mars they will needed to spend some time re-adapting to gravity, thought, the Martian gravity is only partial as it is 3,71m/s2. [34]

The information about the effects of this duration under microgravity is really small as only one space crew stayed a similar period under this condition, Valeri Vladimirovich Polyakov. Therefore, some illnesses have to be considered. Firstly, the bone degradation will be one the main problem for this kind of trip. However, some secondary problems will appear in relation with the losses of calcium in the bones. On of the principal problem which will be involved with this excess of calcium will be the urolithiasis. This problem on Earth is easy solved but onboard can have some complications which can affect to the correct success of the mission and in the worse case can provoke the death of the astronaut.

In conclusion, it is really important to define in detail this problematic and to find some solutions to avoid them as the prevention. This problematic can be focuses from two different points of view. The first one is if the bone degradation is reduced, the excess of calcium in blood and in the urine will be also reduced; therefore, the urolithiasis could be reduced. The other point of view, it is to define ways to reduce the risk to have urolithiasis on board focusing the solution directly on it.

In order to create an effective countermeasure to the bone degradation is important to define in detail the problematic. It was observed that within the 1-2 week onboard of a spaceflight, the excretion of calcium in the urine begin to increase. The rate and extent of calcium loss in space flight varied considerably from individual to individual and was approximately 1.3-1.5% of total body calcium per month. To reduce the bone degradation is important to define a group of exercise or devices. The main problem is that they are not really probed, though; some Russian cosmonauts did some experiments onboard. Some studies in bed-rest patients showed the importance of lower body negative pressure which helps to reduce the excess of calcium in the urine. Therefore, it could be a good option to reduce the possibility to create calculi. This kind of experiments was done previously on board but to define another kind of problematic which were the orthostatic intolerance and the problems in cardiovascular system. Therefore, this technique will be used newly without a lot of problems. In the following picture it is possible to see the astronaut Jack R. Lousma, Skylab 3 pilot, in the Lower Body Negative Pressure Device (LBNPD). [7,8,18,19,20]

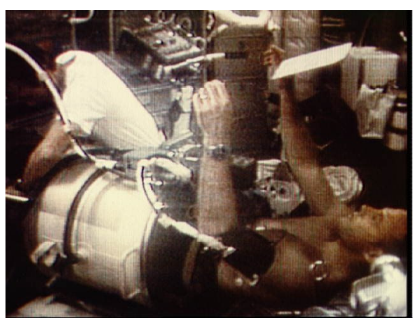


Fig.7.2 Astronaut using LBNPD

From the second option is to analyze the problematic directly from the point of view of urolithiasis, there are some changes on the eating habits which can help to reduce this problematic. The first and easy countermeasure is taking oral fluid to maintain adequate hydratation and to reduce the saturation of calcium in urine. If astronauts suffer from severe space motion sickness, one possibility will be to give them intravenous fluids to avoid deshydration. Another really important measure is to limit the sodium ingestion as it reduces urinary calcium excretion. Moreover, potassium citrate or potassium-magnesium citrate may be useful to reduce the nucleation of calculi. Finally, hormonal and pharmacological treatments can prevent bone resorption. [9-13]

Although, all this prevention methods can help to the reduction of urolithiasis and bone losses, they are not enough to discard this problematic.

7.4. Future work for a manned Mars mission

All the actual countermeasures are not enough efficient and the possibility to suffer urolithiasis in a long mission is still present. The reason why this problem is not solved is because it is not really understood the process of calculi nucleation. This topic is really complex and there are a lot of different hypothesis. One of them, it is the presence of CNP-like particles in the calculi. The problem of this hypothesis is that this bacterium has a bigger multiplication rate under microgravity conditions which can be very relevant, if it is consolidate, for the space mission. [23-30]

To find effective countermeasures to avoid urolithiasis during a long mission, two different kinds of investigations have to be done. The first one is study in more detail this problematic onboard as the information from the space flight and from the ISS is very limited. The other field which has to be studied is the urolithiasis on Earth to define how nucleation is done. When it can be defined, better prevention methods can be created and they will be used on Earth and for long missions.

8. Conclusions

From this project, it is possible to obtain some different conclusions. The first conclusion obtained from the bibliographic research is related to the importance of urolithiasis during space flights. In the period of 2000-2006, the 18% of astronauts suffered urolithiasis. This problem is easy solved on Earth but not onboard. It is important to define solution for it. Moreover, it is possible to confirm that there is not enough information from urolithiasis on board. Finally, the CNP-like particles research shows a lot of different points of view in relation of their origin. Therefore, it is possible to conclude that these particles have a really important role in urolithiasis problematic and also they should be better defined.

From the experimental point of view, it is possible to confirm that in the analysis of the samples' surfaces, there were CNP-like particles. All the samples showed these particles but forming different structures.

The CNP-like particles observed with the atomic force microscope have diameters between 30 and 200nm. This dimension concords with the results obtained from other scientists. However, they defined the smallest ones as they have 50nm of diameter. This difference can appear because the technology used in this project is more precise than the microscope used in the other studies. Therefore, it is possible to observe smaller CNP-like particles.

On the other hand, there are some analyzed CNP-like particles which have a bigger diameter. Those were analyzed from the images where the particles were less independent and less clear. Therefore, this diameter can be obtained from an aggregation of CNP-like particles not from an independent one.

Moreover, they were located like independent particles but also like aggregations. This is one of the characteristics that all scientists which study this kind of particles agree.

To verify the presence of this particles the analysis was done again but with another technique which was the field emission microscope. The results obtained, confirm that there were particles in the surface of the samples which have more or less the same diameter.

Finally, doing a study of the Mars reference mission, it was possible to observe that the presence of urolithiasis is not considered in a possible future mission to this planet. Therefore, it is possible to affirm after finishig this project that it is really important to take in consideration urolithiasis during a long mission.

9. Glossary

Bladder calculi: Calculi of the urinary bladder; also known as vesical calculi, bladder stones or gravel, and cystoliths. Vesicoprostatic calculi are prostatic calculi extending into the bladder.
Body mass index: A key index for relating a person's body weight to their height. The body mass index (BMI) is a person's weight in kilograms (kg) divided by their height in meters (m) squared.
Calcaneus: is a bone of the tarsus of the foot which constitutes the heel.
Femoral neck: the part of the thigh bone that connects to the hip joint.
Forniceal rupture:
Genitourinary: the parts of the body that play a role in reproduction, getting rid of waste products in the form of urine, or both.
Hematuria: Any condition in which the urine contains blood or red blood cells.
Hyperparathyroidism: refers to overactivity and growth of the parathyroid gland or glands.
Lower body negative pressure: External decompression applied to the lower body.
Lower urinary tract: part of the urinary system which includes the bladder and the urethra.
Microgravity: a condition almost equal to weightlessness, as on board the ISS.
Nasal stuffiness: A sensation of difficulty in nasal breathing, associated with increase of nasal airway resistance.
Pathogenesis: the origination and development of a disease.
Sarcoidosis: a systemic disease involving the lungs, lymph nodes, skin, liver, spleen, eyes, phalangial bones, and parotid glands, characterized by granular nodules. Its cause is not known.
Space motion sickness: disorder characterized by nausea, vomiting, and dizziness associated with space flight.
Subepithelial: Occurring or situated beneath an epithelial layer. Subcutaneous.
Supine position: it is a position of the body; lying down with the face up.
Trochanter: one of the bony prominences developed near the upper extremity of the femur to which muscles are attached.
Upper urinary tract: part of the urinary system which includes the kidneys and the ureters.
Urinary retention: Inability to empty the bladder.
Urinary tract: The organs of the body that produce and discharge urine.
Urosepsis: sepsis caused by bacteria from the urinary tract invading the bloodstream.

10. List of acronyms

AFM: atomic force microscope.
CCD: Charge-Coupled Device
CNP: calcifying nanoparticles.
FESEM: Field emission scanning electron microscopy.
VSI: vertical scanning interferometer
LBNPD: Lower Body Negative Pressure Device

11. References

[1] Djojonegoro B., Jones J. (2006) Kidney Stones. In space flight, The Longitudinal Study of Astronaut Health Newsletter , Vol. 14, Issue 2
From: lsda.jsc.nasa.gov/refs/LSAH/Vol_14_Issue_2_Dec_06.pdf
[2] Monga M., Macias B., Groppo E., Kostelec M., Hargens A. (2006) "Renal stone risk in a simulated microgravity environment: impact of treadmill exercise with lower body negative pressure". *The Journal of Urology* 176, Issue 1, pp. 127-131.
[3] Wein, A. J., Kavoussi, L. R., Novick, A. C., Partin, A. W., & Peters, C. A. (Eds.). (2007). *Campbell-walsh urology* (9th ed.) Saunders/Elsevier.
[4] Brenner, B. M. (Ed.). (2004). *Brenner & rector's the kidney* (7th ed.) Saunders.
[5]S Robert W. Schrier (Ed.). (2007). *Diseases of the kidney & urinary tract* (8th ed.) Lippincott Williams & Wilkins.
[6]T Savitz, G. R., & Leslie, S. W. (Eds.). (1999). *The kidney stones handbook: A patient's guide to hope* Four Geez Press.
[7] Whedon, G. D., & Rambaut, P. C. (2006). "Effects of long-duration space flight on calcium metabolism: Review of human studies from skylab to the present". *Acta Astronautica, 58*(2), 59-81
[8] Smith, S. M., & Heer, M. (2002). "Calcium and bone metabolism during space flight". *Nutrition, 18*(10), 849-852.
[9] Zerwekh, J. E. (2002). "Nutrition and renal stone disease in space". *Nutrition, 18*(10), 857-863.
[10] Lane, H. W., & Feeback, D. L. (2002). "History of nutrition in space flight: Overview". *Nutrition, 18*(10), 797-804.
[11] Vodovotz, Y., Smith, S. M., & Lane, H. W. (2000). "Food and nutrition in space: Application to human health". *Nutrition, 16*(7-8), 534-537.
[12] Convertino, V. A. (2002). "Planning strategies for development of effective exercise and nutrition countermeasures for long-duration space flight". *Nutrition, 18*(10), 880-888.
[13] Doty, S. E., & Seagrave, R. C. (2000). "Human water, sodium, and calcium regulation during space flight and exercise". *Acta Astronautica, 46*(9), 591-604.
[14] Barratt, M., & Pool, S. (Eds.). (2008). "*Principles of clinical medicine for space fligh"t* Springer.
[15] Jones, J. A., Jennings, R., Pietryzk, R., Ciftcioglu, N., & Stepaniak, P. (1999). "Genitourinary issues during spaceflight: A review. *International Journal of Impotence Research", Official Journal of the International Society for Impotence Research, 17*, S64-S67.
[16] Ball, J., & Evans, C. H. (Eds.). (2001). *Safe passage: Astronaut care for exploration missions"* (illustrated ed.) National Academies Press.
[18] Miyamoto, A., Shigematsu, T., Fukunaga, T., Kawakami, K., Mukai, C., & Sekiguchi, C. (1998). "Medical baseline data collection on bone and muscle change with space flight". *Bone, 22*(5, Supplement 1), 79S-82S.
[19] Holick, M. F. (1998). "Perspective on the impact of weightlessness on calcium and bone metabolism". *Bone, 22*(5, Supplement 1), 105S-111S.
[20] Zérath, E. (1998). "Effects of microgravity on bone and calcium homeostasis". *Advances in Space Research, 21*(8-9), 1049-1058.

[21] Slavković A, Vlajković M, Radovanović M, Sirić Z, Stefanović V. (2001). "Combined treatment of immobilization staghorn stones in 14-year old boy". *International Urology and Nephrology, 32*(4), 597-599.
[22] Whitson PA, Pietrzyk RA, Morukov BV, Sams CF, (2001) "The Risk of Renal Stone Formation during and after Long Duration Space Flight", *Nephron;*89:264-270
[23] Çiftçioglu, N.; Björklund, M.; Kuorikoski, K.; Bergström, K.; Kajander, E. O. (1999), "Nanobacteria: An infectious cause for kidney stone formation", *Kidney International*, 56(5), (1893-1898).
[24] Ciftçioglu, N. Nanobacteria-Discovery of a new form of life. *Horizons* 2006 March 31: 7-8.
[25] Kajander EO. (2006) "Nanobacteria - propagating calcifying nanoparticles". *Lett Appl Microbiol*. 42(6):549-52.
[26] Ciftcioglu, N., R. S. Haddad, D. C. Golden, D. R. Morrison, D. S. McKay. (2005) "A potential cause of kidney stone formation during space flights: Enhanced growth of nanobacteria in microgravity". *Kidney International.* 67: 483-491
[27] Jones, J. A., Ciftcioglu, N., Schmid, J. F., Barr, Y. R., & Griffith, D. "Calcifying nanoparticles (nanobacteria): An additional potential factor for urolithiasis in space flight crews". *Urology, In Press, Corrected Proof*
[28] Aloisi, G. (2008). "An alternative origin for nanobacteria in kidney stones". *Bioscience Hypotheses, 1*(3), 138-141.
[29] Martel J, Young JD. (2008), "Purported nanobacteria in human blood as calcium carbonate nanoparticles". Proc Natl Acad Sci U S A. 8;105(14):5549-54
[30] Wood, H.M., Shoskes, D.A. 2006. "The role of nanobacteria in urologic disease. *Journal of Urology* 24(1):51-4.
[31] Olszak, A.G., Heaton, M.G., "Interferometry: Technolgy and Applications", Veeco metrology group
[32] Mozer, W. "Atomic force microscopy: Technology and Applications", Veeco metrology group
[33] D S Joag, field emission microscope, *Department of Physics University of Pune, From: http://physics.unipune.ernet.in/~fem/intro-fem.htm*
[34] Hoffman S.J, Kaplan D. L., (1997) "Human Exploration of Mars: The Reference Mission of the NASA Mars Exploration Study Team", Lyndon B. Johnson Space Center Houston, Texas
[35] Portree D. S. F., (2001) *Humans to Mars: Fifty Years of Mission Planning, 1950 - 2000,* NASA Monographs in Aerospace History Series, Number 21, February
[36] Drake B. G. (1998) "Addendum (Update) to Human Exploration of Mars: The Reference Mission of the NASA Mars Exploration Study Team", Johnson Space Center. Houston, Texas.